Functional Consequences and Regulation of Hypoxia-Inducible Factor-2α in Human Breast Cancer

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Summary

Breast cancer is one of the most common cancers worldwide and the most common cancers in women. As in other types of cancer, breast cancer is a multi-factorial disease, consisting of various internal and external factors influencing the development and progression of the disease. Hypoxia and estrogen signalling have been established to be involved in the development of breast cancer.

Hypoxia plays a crucial role in tumour development and metastasis. The key regulator in cellular hypoxic adaptation is hypoxia-inducible factor (HIF), which consists of the constitutively expressed β-subunit and the oxygen-sensitive α-subunit. HIF-α can be classified into three different isoforms: HIF-1α, HIF-2α and HIF-3α, among which HIF-2α is one of the less well characterised isoforms. Although HIF-2α has long been reported, the functional consequences and regulation of HIF-2α are not well understood. Wnt-1-inducible signalling protein 2 (WISP-2) is one of the few known genes to be selectively induced by HIF-2α. Clinically, patients expressing both WISP-2 and AREG have been reported to possess significant overall survival. Four hypoxia response elements (HREs) were discovered within the *WISP-2* promoter, in which two HREs were necessary for WISP-2 regulation by HIF-2α. Moreover, the two active HREs are located within microsatellite (MS) regions which have been established to play an essential role in tumour progression. A negative correlation between WISP-2 expression and tumour macrophage numbers was observed, supporting the better prognosis of patients expressing WISP-2, as lower macrophage infiltration means lower cancer aggressiveness. Furthermore, proliferation, anchorage-independent colony formation, and scratch recovery assays suggest the reduction in the tumourigenic properties of MCF-7 breast cancer cells, in line with the tumour-suppressor like characteristic of WISP-2 and its regulation by HIF-2α.

Besides hypoxia, the involvement of estrogen (E2) in the development and progression of breast cancer is well established. In this study, treatment of E2 on estrogen receptor (ER)-positive breast cancer cell lines resulted in HIF-2α mRNA and protein downregulation. The involvement of ER was confirmed with the absence of HIF-2α downregulation upon treatment of E2 on ER-negative breast cancer cell lines. Moreover, the role of ERα in the downregulation was further established by utilising both pharmacological and siRNA approaches. An investigation of 690 samples from breast cancer patients revealed the association of HIF-2α tumour levels with a better prognosis in the triple-positive patients, which was validated with less pronounced HIF-2α downregulation on a triple-positive breast cancer cell line, BT474. To explore the potential molecular mechanism, trichostatin A (TSA), a histone deacetylate (HDAC) inhibitor, was added together with E2. The blunting of HIF-2α downregulation in the presence of TSA indicated the transcriptional origin of HIF-2α downregulation. *In silico* analysis revealed the presence of four bound estrogen response elements (EREs) within the first intron of *EPAS1*, in which one ERE was activated upon treatment with E2.

In conclusion, this thesis reports functional consequences of HIF-2α in human breast cancer by its regulation of WISP-2. Furthermore, the novel regulation of HIF-2α by estrogen signalling was described, which might partially explain the association of high HIF-2α in triple-positive breast cancer patients with better prognosis.

Zusammenfassung

Weltweit ist Brustkrebs eine der häufigsten Krebserkrangkungen und die häufigste bei Frauen. Wie bei anderen Typen von Krebs, ist Brustkrebs eine vielfältige Krankheit, bestehend aus inneren und ausseren Faktoren, die die Entwicklung und das Fortschreiten der Krankheit beeinflussen. Aus der molekularen Perspektive sind die beiden Faktoren Hypoxie und der Östrogen-Signalweg, welche an der Entwicklung des Brustkrebs beteiligt sind, gut untersucht.

Hypoxie ist wichtig für die Entwicklung des Tumors und die Metastase. Der Hauptregulator in der Anpassung an zelluläre Hypoxie ist der Hypoxie-induzierbare Faktor (HIF), der aus einer konstitutiv exprimierten β-Untereinheit und einer Sauerstoff-sensitiven α-Untereinheit besteht. Diese tritt in drei unterschiedlichen Isoformen auf. Darunter stellt HIF-2α eine der weniger erforschten Untereinheiten dar. Insbesondere die HIF-2α Regulierung und deren funktionelle Auswirkungen sind bisher kaum untersucht worden. Das Wnt-1-induzierbare Signal Protein 2 (WISP-2) ist eines der wenigen Gene, welches durch HIF-2α selektiv induziert wird. Patienten mit einer erhöhten WISP-2 und Amphiregulin (AREG) Expression haben klinisch gesehen eine höhere Überlebensrate. Innerhalb des WISP-2 Promotors wurden vier Hypoxie responsive Elemente (HREs) entdeckt, von denen zwei für die HIF-2α abhängige WISP-2 Regulierung zuständig sind. Die beiden HREs liegen in einer Mikrosatellitenregion (MS), welche bei der Tumorentwicklung eine wichtige Rolle spielt. Des weiteren konnte eine negative Korrelation zwischen der WISP-2 Expression und der Anzahl an Makrophagen Tumor-assoziation festgestellt werden. Eine geringere Infiltration der Makrophagen geht mit einem weniger aggressiven Krankheitsverlauf einher. Dies unterstützt die Beobachtung, dass Patienten mit einer erhöhten WISP-2 Expression eine bessere Prognose erhalten. Ausserdem zeigten die Zellproliferation, Ankergrund-unabhängige Kolonie formierung, und „Scratch“ Versuche eine Senkung der Tumor-Eigenschaften durch WISP-2 in MCF-7 Brustkrebs Zellen. Dies bestätigt die Tumorsuppressorcharakteristik des WISP-2 und seine Regulierung durch HIF-2α.

Ausser Hypoxie ist die Rolle des Östrogens (E2) in der Entwicklung und dem Fortschreiten des Brustkrebs schon gut bekannt. Experimente mit Östrogenrezeptor (ER)-positiven Brustkrebszelllinien, welche mit E2 behandelt wurden, wiesen auf eine reduzierte Menge an HIF-2α mRNS und Protein hin. Die Mitwirkung des ER konnte mit der Abwesenheit der HIF-2α Herunterregulierung nach der E2 Behandlung von ER-negativen Brustkrebszellen bestätigt werden. Zusätzlich konnte der Effekt von ERα auf die HIF-2α Herunterregulierung pharmakologisch und durch siRNS Methoden belegt werden. Die Analyse von 690 Brustkrebspatientenproben wies auf eine Assozation des HIF-2α Tumorgehalts und einer besseren Prognose der dreifach Rezeptor-positiven Patienten hin. Mit Hilfe der dreifach Rezeptor-positiven Brustkrebszelllinie BT474 konnte die reduzierte Herunterregulierung von HIF-2α nach E2 Behandlung ebenfalls bestätigt werden. Um den potenziellen molekularen

Mechanismus zu untersuchen, der diesem Effekt zu Grunde liegt, wurden die Zellen mit Trichostatin A (TSA), einem Histon Deacetylase (HDAC) Inhibitor, und E2 behandelt. Die Abnahme der HIF-2α Herunterregulierung in der Anwesenheit von TSA deutet darauf hin, dass die Herunterregulierung auf transkriptioneller Ebene stattfindet. Eine *in silico* Analyse zeigte die Anwesenheit von vier Östrogen responsiven Elementen (EREs) im ersten Intron von Endothelial PAS domain-containing protein 1 (*EPAS1*), wovon ein ERE durch die Hinzugabe von E2 aktiviert wurde.

Zusammenfassend wurden im Rahmen dieser Dissertation die funktionellen Zusammenhänge von WISP-2 und dessen Regulierung durch HIF-2α bei der Brustkrebsentwicklung untersucht. Zudem wurde die neu entdeckte E2-abhängige Regulierung von HIF-2α und die Assoziation zwischen dem Gehalt von HIF-2α in dreifach Rezeptor-positiven Brustkrebspatienten und einer besseren Prognose beschrieben.

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1. Breast Cancer

1.1 Introduction

Breast cancer is the most common cancer in women worldwide and the second most common cancer overall, with approximately 1.7 million new cases diagnosed in 2012[1,2]. This number represents 12% of all new cancer cases and 25% of all cancers in women[1,2]. In the United States, breast cancer incidence rates have increased constantly at a rate of 1% per year since 1940[3]. This increase is not limited to developed countries as the incidence is likewise on the rise in many developing countries, including Asian countries that have previously been shown to have a low risk of breast cancer, such as Japan and Singapore[4]. In contrast to the incidence rates, the breast cancer mortality rates have plateaued and in some countries have started to decline[5,6]. The decline of the mortality rates has been attributed to improvements in the screening procedures[6]. Similarly, advances in breast cancer treatment have generated significant but modest survival benefits, contributing to lower mortality rates[6].

Epidemiological observations showed that daughters of women from low-incidence countries who migrate to high-incidence countries acquire the breast cancer risk of the host country, suggesting that environmental and/or behavioural factors play a more significant role in the development of breast cancer than genetic factors[7]. In fact, genetic factors are responsible for at most 5-10% of the breast cancer cases[8]. Various population-based studies have implicated multiple factors involved in breast cancer development. These factors include genetic susceptibility and family history, endogenous steroid hormone levels, age of menarche and menopause, parity, lactation, physical attributes and activity, social and dietary habits, and exposure to environmental pollution[9]. However, it is still uncertain whether these factors cause breast cancer. As such, the fundamental cause of breast cancer development remains unknown.

1.2 Breast development

The breast is a bilateral organ overlying the chest (pectoral) muscles and is made of specialised tissue that produces milk (glandular tissue) and fatty tissue, which determine the size of the breast[10]. The glandular tissue of female breast is made up of two different structures: the lobes and the ducts, with each breast consisting of approximately 15 to 20 lobes **(Figure 1.1)**[10-12]. Each lobe is made up of numerous smaller sections termed lobules, which in turn consist of tiny bulbs which produce milk[13,14]. The three structures are then linked by thin tubular ducts.

In female, breast progressively undergoes changes in association with infantile growth, puberty, pregnancy, lactation, and post-menopausal regression[15,16]. Human breast development is a life-long process which starts during embryonic life, followed

by the major growth spurt with lobular development during puberty and finally with the completion of the development and differentiation by the end of the first full-term pregnancy[17]. Case control studies have shown an inverse relationship between the risk of breast cancer and early parity, especially that the risk of breast cancer increases with the age at which a woman experiences full-term pregnancy[18]. The protective factor in these observations appears to be the duration between the age of menarche and the age of first full-term pregnancy[19]. The actual mechanism behind the protective properties of first full-term pregnancy on breast cancer development is yet unknown, but the fact that pregnancy induces breast differentiation led to the speculation that the state of breast differentiation is important for breast cancer development.

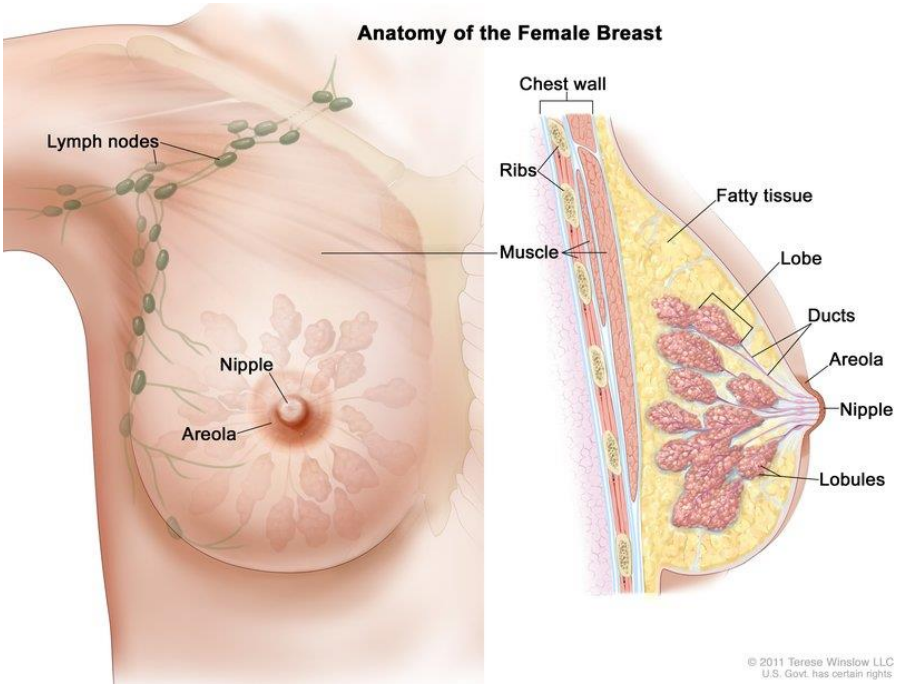

Figure 1.1 Representation of female breast anatomy. Female breast consists of a network of lobes and ducts embedded within fatty tissue and attached to the chest (pectoral) muscles. The nipple and areola are located outside of the breast. (**For the National Cancer Institute © 2011 Teresa Winslow LLC, U.S. Govt. has certain rights)**

1.2.1 Prenatal, perinatal and postnatal development

The mammary gland tissue originates from a single epithelial ectodermal bud and begins during early foetal life, approximately 4 weeks after conception[20]. After birth, mammary gland develops at the same pace as the general growth of the body[21]. The first major development of breast occurs during puberty, with the growth of glandular tissue and the surrounding stroma[20]. Furthermore, the ducts grow and divide to form the terminal end buds (TEBs), which together with the terminal ducts (TD) and the proliferating ductules form the first identifiable lobular structure termed the lobule type 1 (Lobule 1) or terminal ductal lobular unit (TDLU)[22]. The breast tissue in a non-pregnant female with continuous cycles of sex steroid exposure consists of three

identifiable structures, the aforementioned Lobule 1, the more developed lobule type 2 (Lobule 2) and the further developed Lobule 3[22].

As the female undergoes sexual maturation the percentages of the three lobular types change[23]. During sexual maturation, Lobule 1 undertakes gradual sprouting of new ductules and eventually formed a denser lobular structure termed the Lobule 2[23]. The breast reaches its maximum development during pregnancy; active proliferation of the ductal tree occurs, rapidly increasing the number of ductules generated and results in the conversion of Lobule 2 into Lobule 3 **(Figure 1.2)**[15]. The breast is then fully differentiated during lactation with the formation of Lobule 4 before undergoing post-lactational involution[15]

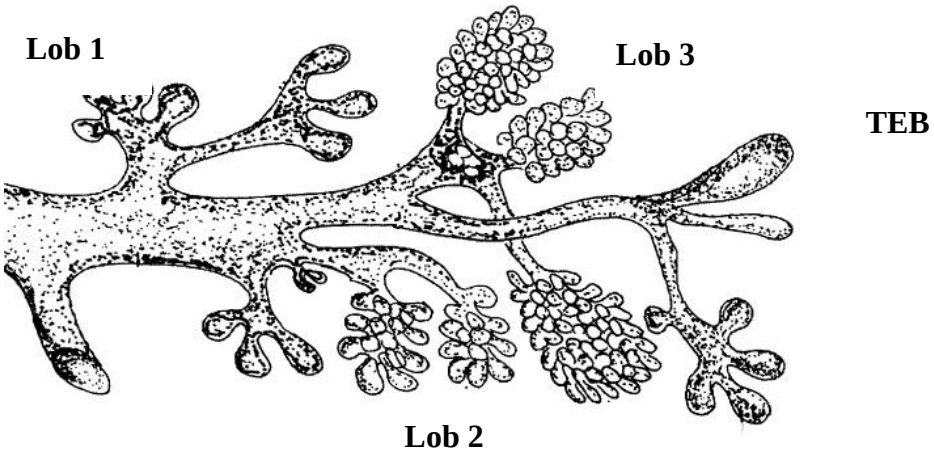

Figure 1.2 Representation of lobular structures in the breast. The diagram shows the lobular structures of non-lactating female breast. TEB: terminal end buds, Lob 1: lobule type 1, Lob 2: lobule type 2, Lob 3: lobule type 3. (Adapted from Russo and Russo, 2004[15])

The influence of parity can be observed from the development and the distribution of the different types of lobules within the breast[23]. In nulliparous female, the percentage of Lobule 1 remains constant, with modest increase in the percentage of Lobule 2 during the early reproductive years and sharply decreasing after the age of 23, and complete absence of Lobule 3[23]. This observation of the difference in the composition of lobules between parous and nulliparous females suggests that several Lobule 1 might have progressed into Lobule 2, but no Lobule 2 have progressed to Lobule 3[23]. In combination with the fact that the more differentiated lobules possess lower proliferation activities; this might be a possible explanation for the higher risk of breast cancer in nulliparous women.

1.2.2 Hormonal regulation of breast development

The breast is a hormone-sensitive organ whose development is influenced by a myriad of hormones and growth factors, affecting cell proliferation, cell differentiation and apoptosis[24-26]. Among the multiple hormones influencing the development of breast, estrogens are considered to be the major hormones by promoting the proliferation of both normal and neoplastic breast epithelium[24,27]. Estrogens act

locally on the mammary gland and stimulate DNA synthesis, cell proliferation and bud formation[28,29]. In a classical model of estrogen actions, the binding of estrogens to their receptors (ERα and ERβ) results in the formation of a homodimer with the corresponding other ligand-activated ER, followed by nuclear translocation and transactivation of downstream target genes containing estrogen response elements (EREs)[30]. Another steroidal hormone with important, albeit controversial implications in mammary gland development is progesterone[31-33]

The role of progesterone in breast development is controversial due to the fact that progesterone receptor (PgR) is a known downstream target of estrogen signalling, and hence the difficulties in differentiating the action of estrogen signalling and progesterone signalling in breast development. There have been multiple reports describing the role of estrogen as a stimulant for cell proliferation, however, the breast epithelium of sexually mature and normally cycling female does not exhibit maximal proliferative activity during the follicular phase of the menstrual cycle, when estrogen is at its peak levels (200 pg/ml serum compared to progesterone < 1 ng/ml serum)[34,35]. Instead, the breast epithelium reaches its maximal proliferation during the luteal phase, when progesterone reaches its peak levels of 10-20 ng/ml serum, while estrogen levels drop to 2- or 3-fold lower than during the follicular phase, suggesting the possible cooperation between estrogen and progesterone signalling in breast development[35]

Besides the responsiveness of breast epithelium to circulating hormones, the degree of differentiation of the mammary parenchyma also plays a role in regulating the proliferation of breast epithelium in both rodents and humans[17,36]. In human, the highest level of cell proliferation occurs in the non-differentiated cells of Lobule 1[37]. Interestingly, the highly proliferating cells within the non-differentiated Lobule 1 also possess the highest amounts of ERα and PgR[38]. Furthermore, the content of ERα and PgR together with the proliferation rate is decreasing progressively in the more differentiated Lobule 2, Lobule 3, and Lobule 4, suggesting the role of both estrogen and progesterone signalling in breast development[38]. As breast cancer development is often associated with increasing proliferation, these observations provide additional rationale for the higher risks of breast cancer in nulliparous female compared to parous female, due to the higher proportion of non-differentiated Lobule 1 in nulliparous female and the higher content of ERα and PgR[38].

1.3 The pathogenesis of breast cancer

Breast development, which occurs during the period of female sexual maturation between menarche and first full term pregnancy, represents a period of high susceptibility for the initiation of breast cancer. The classification of the lobular structures of breast into the non-differentiated Lobule 1 to the fully differentiated Lobule 4 has assisted in describing the site of origin of breast cancer[23]. Observations of breast development have identified the TDLU as the site of origin of the ductal

carcinoma, which is highly associated with the abundance of Lobule 1[39]. Furthermore, other lobular structures have been identified as sites of origin for specific types of breast carcinoma: Lobule 2 is the site of origin for lobular carcinoma, Lobule 4 originates hyperplastic lobules, cysts, fibroadenomas, and adenomas, and Lobule 4 give rise to lactating adenoma[40].

From observing breast development, a concept emerged postulating that breast architecture is an important factor in determining the susceptibility of the human breast to cancer[23,41]. The breast composition between nulliparous and parous females differ regarding the presence of the non-differentiated Lobule 1 and the more differentiated Lobule 2 and Lobule 3, with nulliparous female exhibiting higher proportion of Lobule 1 and vice versa for parous female[23]. Together these observations suggest that breast cancer originates from the non-differentiated terminal structures of the mammary gland[23]. Furthermore, the Lobule 1, which is the site of the most common carcinoma, the ductal carcinoma, proliferates at its maximal rate during early adulthood, coincides with the highest susceptibility of the breast to carcinogenesis, providing a rationale in identifying genes controlling breast development for breast cancer prevention[42].

1.4 Genomic changes in breast cancer development

Breast cancer, as other types of cancer, is a genetic disease and it has been reported that genetic factors contribute to approximately 5-10% of breast cancer cases[8]. Two types of genetic alterations might contribute to the initiation and development of breast cancer: microsatellite instability (MSI) and loss of heterozygosity (LOH).

1.4.1 MSI and LOH in breast cancer development

Microsatellites are repeated sequences (approximately 5-50 repeats) of DNA with lengths ranging from 2-5 base pairs. Microsatellites are presence in multiple locations in the human genome and are known for their diversity within the population[43]. MSI is a hypermutable phenotype and reflects the presence of defective DNA replication processes and the loss of DNA mismatch repair mechanism[43]. The MSI phenotype is common in some colon, gastric, pancreatic and endometrial cancer[44-46]. It is detected in 15% of colorectal cancer; however its occurrence in breast cancer has been so far inconsistently reported[44,47,48]. Several observations have supported the involvement of MSI in breast cancer initiation. MSI has been reported to be positively correlated with the progression of human breast carcinoma in situ into invasive carcinoma[49]. *In vitro* studies have also shown an association between the presence of MSI and the immortalisation and progressive transformation of non-cancerous human breast epithelial cells (HBECs)[50].

LOH is a chromosomal event, in which an individual possesses only one functionally active allele. LOH increases the chance of cancer development in an individual and it

is the most common genomic alterations observed in human cancers[51]. LOH is a molecular representation of aneuploidy, which is a common feature of all cancer[52]. This type of genetic alteration might contribute to cancer development via deletion of tumour suppressor genes and dysregulation of general gene expression, which leads to abnormal growth control[53-55].

1.4.2 Genes involved in breast cancer development

Normal HBECs have limited proliferative activity and a common characteristic of all cultured HBECs is their limited life span *in vitro*[56]. Under standard culture medium maintenance, HBECs undergo approximately 30-40 times population doublings before terminal differentiation and senescence, comparable to adult human fibroblasts[56]. From the *in vitro* perspective, immortalisation of cells represents an initial step in the development of breast carcinoma, which is comparable to the ductal hyperplasia or the pre-neoplastic stage *in vivo*. As such, investigating differential gene expression between non-immortalised HBECs and immortalised HBECs might illuminate genes important in the initiation of breast carcinoma. Two particular genes have been reported to be differentially expressed between non-immortalised HBECs and the immortalised HBEC cell line MCF-10F[57,58]. The two genes are ferritin H chain and S100p[57,58].

The ferritin H chain is an important component of cellular iron transport and its expression is associated with intake of iron[59]. Intake of iron and its associated transport proteins have been reported to play a significant role in cell proliferation by influencing DNA synthesis via its activity on the enzyme ribonucleotide reductase[60]. The increase in proliferation might assist in the development of breast carcinoma[61]. Furthermore, increase in intracellular iron content might increase the free radical reactions leading to oxidative damage and mutation of DNA[62]. Another possible mechanism in which ferritin H chain might contribute to breast cancer is the role of ferritin as modulator of immune response by modulating the maturation of B and T lymphocytes and providing growth advantage for breast cancer cells[63,64].

The S100P calcium-binding protein is highly expressed in breast cancer compared to the adjacent normal tissues[65]. S100P is a member of the so-called S100 calcium-binding proteins, which play an important role in maintaining intracellular calcium concentration[66]. S100P has been reported to be a prognostic factor for hepatocellular carcinoma, colorectal cancer, gastric cancer and breast cancer[67-71]. The dysregulation of intracellular calcium maintenance with the overexpression S100P might result in dysfunctional assembly of microtubules, which in turn leads to an increase in motility and invasive property of cells[72]. Additionally, calcium can act as secondary messenger, which activates the immediate early genes responsible for inducing resting cells to re-enter cell cycle and in turn affects the proliferation of cells[73].

Another gene, which when mutated is almost always associated with carcinogenesis, is p53[74]. The p53 protein is a nuclear phosphoprotein possessing anti-proliferative,

cell cycle-regulatory, and apoptotic inducing properties[75-77]. As such, it has been identified as tumour suppressor gene. The p53 gene is often mutated in breast carcinoma, associated with an increase in genomic instability and further mutations of proto-oncogenes into oncogenes, such as *ras, c-myc,* and *mdm*[78-81].

1.5 Treatment of breast cancer

Currently there are five standard treatments of breast cancer patients: surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy.

1.5.1 Surgery

Surgery has been the procedure of choice for the treatment of breast cancer for centuries, with the first publication of a radical mastectomy in 1894[82]. The basic principle underlying surgery as a method of treatment is removal of cancerous or potentially affected breast tissue. This will convey maximum protection and remedy from breast cancer. Three types of surgery are currently available: breast-conserving mastectomy, total mastectomy, and modified radical mastectomy[83].

As the name suggests, breast-conserving mastectomy is a type of mastectomy in which cancer and some normal tissues surrounding it are removed except for the breast itself. It is also often termed as lumpectomy, partial mastectomy, segmental mastectomy, quadrantectomy, or breast-sparing surgery. Total mastectomy or simple mastectomy is the total removal of the breast with cancer. And lastly, modified radical mastectomy is a type of surgery, in which the whole breast with cancer, the lymph nodes under the arm, the lining of the chest muscles and part of the chest wall muscles are removed.

1.5.2 Chemotherapy

Chemotherapy is a type of cancer treatment in which one or more chemotherapeutic agents are introduced to the patients to stop the growth of cancer cells, either by killing the cells or preventing cell division. This treatment works as cancer cells proliferate faster than normal cells. Two types of chemotherapy regimen are currently available: anthracycline-based regimens and methotrexate-based regimens[84]. The anti-tumour activity of anthracycline is based on the ability of the antibiotics to intercalate between DNA and RNA base pairs and hence blocking DNA replication and transcription[85]. Furthermore, anthracycline also inhibits topoisomerase II enzyme, which is required for DNA transcription and replication[86]. Meanwhile, methotrexate acts by inhibiting folic acid reductase, an enzyme required for DNA synthesis and cellular replication[87]. The anthracycline-based regimens have been reported to be clinically more effective than methotrexate-based regimens[88]. However, the anthracycline-based regimens have more adverse side effects, with association between anthracycline with heart failure reported in older patients with history of cardiac diseases[89].

Chemotherapy may also be given to patients before mastectomy in a procedure termed neo-adjuvant therapy[90]. The rationale behind this pre-treatment is to reduce the size of the tumour and hence reducing the amount of tissue that needs to be removed during mastectomy.

1.5.3 Radiotherapy

In radiotherapy, ionising radiation is applied to control or kill malignant cells. Ionising radiation works by inducing DNA damage and subsequently cellular death. Cancerous cells proliferate at a faster rate compared to normal cells and hence the higher likelihood of the ionising radiation targeting cancer cells.

Radiotherapy is applied as either a curative therapy for localised tumours or as part of adjuvant therapy to prevent recurrence of tumour. Generally, radiotherapy is offered for women with recurrent cancer who had no prior radiation. The treatment protocol consists of whole-breast radiation for 6 – 6,5 weeks[83]. Radiotherapy is often used in conjunction with chemotherapy in a combination therapy called chemo-radiotherapy[91,92].

1.5.4 Endocrine therapy

The importance of hormones in carcinogenesis has long been known since prolonged exposure to female sex hormones has been associated with the development and progression of breast, vaginal, hepatic and cervical carcinomas[93-95]. Furthermore, a vast majority of breast cancers are initially hormone-dependent and especially estrogen-dependent[96]. These provide a rationale for endocrine therapy, a cancer treatment in which hormone actions are blocked to prevent cancer cells from proliferating.

Tamoxifen is currently the first-line treatment for all stages of breast cancer as recommended by the World Health Organisation (WHO)[97]. Tamoxifen is a triphenylethylene anti-estrogen which is often given to patients with early, surgically removable, localised breast cancer and patients with metastatic breast cancer[98]. However, patients using tamoxifen possess higher risk of developing endometrial cancer[99]. As second-line treatment, aromatase inhibitors are used for the treatment of advanced breast cancer. Aromatase inhibitors, such as fadrozole, vorozole and liarozole, are often given to post-menopausal female with ER-positive breast cancer[100]. Unlike tamoxifen, which blocks the activation of ER, aromatase inhibitors inhibit the enzyme aromatase which is involved in the conversion of androgens into estrogens. However, aromatase inhibitors should not be given to pre-menopausal females, since aromatase inhibitors activate the hypothalamic-pituitary-adrenal axis with the consequence of increase androgen production[101].

1.5.5 Targeted therapy

Targeted therapy can technically be considered as “chemotherapy” as it is also a type of treatment in which drugs or other substances are introduced to kill cancer

cells. However unlike traditional chemotherapy drugs which target rapidly dividing cells by interfering with general cell division processes, such as DNA replication and microtubule assembly, targeted therapy drugs identify and interfere with specific molecules critical for tumour progression without affecting normal cells, normally using specific antibodies against the molecules involved. The specificity of targeted therapy drugs results in the same or even more effective treatment compared to radiotherapy and chemotherapy, and additionally with less harmful side effects to healthy cells.

Currently there are two main types of targeted therapies: monoclonal antibody therapy and small-molecule therapy[102]. The introduction of trastuzumab (anti-Herceptin monoclonal antibodies), which blocks the effects of the growth factor HER2, has heralded the arrival of targeted therapy as one of several treatment options for breast cancer patients[103]. Other currently available targeted therapy drugs for breast cancer treatment include Pertuzumab and Ado-trastuzumab emtansine, which is an antibody-drug conjugate used to treat HER2-positive breast cancer that has metastasised[104].

Besides antibody-based drugs, targeting specific proteins unique to cancer cells, small-molecule drugs are also used as targeted therapy drugs. Small-molecule drugs target proteins required for cell proliferation, such as tyrosine kinases (lapatinib) and cyclin-dependent kinases (palbociclib); or DNA repair and apoptosis, such as poly (ADP-ribose) polymerase (PARP)[105-107].

2. Hypoxia

2.1 Introduction

Oxygen is essential for the maintenance of life of all eukaryotes, including unicellular organisms, plants and animals. During evolution, early organisms developed the requisite tools, such as chlorophyll pigment which eventually developed into photosynthetic chloroplast, to convert carbon dioxide (CO_2) and water (H_2O) into glucose ($C_6H_{12}O_6$) and oxygen (O_2)[108]. The spread of these photosynthetic organisms brought about the rise of O_2 concentration in the atmosphere, which eventually led to the appropriate environment for metazoan evolution[108].

The importance of oxygen for the maintenance of life is mainly due to the role it plays as final electron acceptor in the mitochondrial respiratory chain[108]. By accepting electrons, oxygen allows the oxidative phosphorylation process to proceed. This subsequently leads to a more efficient generation of adenosine triphosphate (ATP), the currency for intracellular energy transfer; with 30 molecules of ATP per glucose compared to 2 molecules of ATP per glucose in anaerobic glycolysis[109]. Under normoxia, the relatively high ATP/ADP ratio is maintained by the availability of oxygen and the continuous generation of ATP via oxidative phosphorylation, hence the dependence of cells on oxygen[109].

Hypoxia is defined as an imbalance between oxygen supply and consumption leading to a reduction in oxygen to a level insufficient to maintain normal cellular function[110]. In multicellular organisms, such as *Homo sapiens*, hypoxia occurs in both physiological and patho-physiological conditions[110]. Hypoxia has been reported to play essential roles in embryonic development, cancer, atherosclerosis, and ischemic disease[111-114]. Due to the essentiality of oxygen in human physiology, a mechanism must exist in order to sense the level of oxygen and to induce adaptation processes to cope with changes in oxygen level.

2.2 Adaptation to hypoxia

The absolute requirement of oxygen in the survival of most living organisms necessitates adaptation processes in response to changes of environmental oxygen level. Fundamentally, adaptation to hypoxia can be classified based on the time of responds: acute responses (occurring over a time period of seconds to minutes) and chronic responses (occurring over a longer time period of several hours to days).

2.2.1 Acute responses to hypoxia

The deficiency of oxygen is a major factor in the pathogenesis of numerous diseases, which might lead to the death of the organism. In multicellular organisms, particularly in mammals, responses to acute hypoxia require rapid systemic respiratory and local

cardiovascular regulations in order to ensure sufficient oxygen delivery to critical organs such as the brain and the heart.

The changes in oxygen partial pressure are also sensed by glomus cells within the aortic and carotid bodies[115]. Glomus cells (type I) are peripheral chemoreceptors, which can sense a decrease in oxygen partial pressure (pO_2) and increase in carbon dioxide partial pressure (pCO_2), a parameter tightly associated with decreased blood pH[115]. The activation of the cardiorespiratory centre of the medulla leads to an acute and reversible increase in ventilation in order to transport more oxygen to the lungs[116,117]. This acute increase in ventilation is followed by a ventilator decline and then a slow increase in ventilation which occurs during the acclimatisation period[116,117]. The decrease in pO_2 induces increases in heart rate and cardiac output in order to deliver more oxygen to the peripheral tissues[115]. Subsequent responses include systemic arterial vasodilation and pulmonary vasoconstriction, particularly in the coronary and cerebral vessels[115]. In summary, a decrease in oxygen level induces physiological changes to compensate for the lower oxygen level by activating processes that increase the availability of oxygen.

Cellular responses of type I glomus cells to acute hypoxia depend on the activity of excitable cells with oxygen-dependent ion channels, which modulate the excitability and secretory activity of the cells upon changes in pO_2[118]. The excitation of cells by hypoxia relies on the open probability of membrane K^+ channels which is decreased by low pO_2[119]. In essence, low pO_2 induces the closure of K^+ channels, which subsequently leads to membrane depolarisation, opening of voltage-gated Ca^{2+}channels, influx of Ca^{2+}, release of transmitters, including ATP, dopamine and acetylcholine, into the synaptic cleft and activation of afferent nerve fibres[119,120].

2.2.2 Chronic responses to hypoxia

Contrary to acute responses, chronic exposure to hypoxia results mainly in the induction of gene expression, which facilitates the switch of ATP generation from oxygen-dependent oxidative phosphorylation to oxygen-independent glycolysis, increases the oxygen-carrying capacity of blood, and increases vascularisation for better transport of oxygen to tissues[121].

One fundamental way in which cells adapt to chronic exposure of hypoxia is to increase the usage of glucose via non-oxygen dependent mechanisms[122]. In most cells, hypoxia induces the expression of glucose transporters and glycolytic enzymes[123]. Glucose transporter 1 (GLUT1) is a transport protein which facilitates the transport of glucose across the plasma membrane[124]. Other enzymes, whose expression is induced by hypoxia, include aldolase A, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, phosphofructokinase, pyruvate kinase, and triphosphate isomerase[125-130].

Additionally, O_2 partial pressure of blood also induces the expression of erythropoietin (EPO)[131]. EPO is a glycoprotein hormone which acts as a cytokine for erythrocyte (red blood cells) precursors in the bone marrow[132]. As such, EPO is the

primary factor in erythropoiesis, a process in which erythrocytes are produced[132]. The sites of EPO production are in peritubular fibroblast-like cells of adult kidney and in hepatocytes of foetal and adult liver, which sense changes in tissue pO_2 and respond by inducing the transcription of EPO[132]. EPO is subsequently released into the blood stream and stimulates proliferation and differentiation and prevents apoptosis of the erythrocyte precursors in the bone marrow. Erythrocytes are the major blood oxygen transporters due to the content of haemoglobin within their cytoplasm. As such, induction of erythrocyte production restores the oxygen-carrying capacity of the blood.

Finally, angiogenesis is regulated by changes in oxygen tension both in physiological development and in adaptation to local hypoxia[133]. Several hypoxically-induced cytokines are required for angiogenesis: vascular endothelial growth factor (VEGF), angiopoietin-1, angiopoietin-2, and Tie2[134-137]. The most thoroughly studied angiogenic factor, VEGF, is expressed in nearly all tissues and cells, but its receptor is only expressed in endothelial cells, further supporting its function in inducing angiogenesis.

2.3 The canonical hypoxia signalling pathway

2.3.1 The hypoxia-inducible factor (HIF)

Hypoxia-inducible factors (HIFs) are heterodimeric complexes consisting of two subunits: an oxygen-labile α-subunit and a constitutively expressed β-subunit. HIF proteins are members of the bHLH-PAS (basic helix loop helix-Per/ARNT/Sim) family of TFs[138]. The oxygen-regulated α-subunits are classified into three different isoforms: HIF-1α, HIF-2α, and HIF-3α. All of the three HIF-α subunits are homologous to each other, but while HIF-1α is ubiquitously expressed, HIF-2α and HIF-3α possess a more limited tissue expression profile[139]. Interestingly, HIF-1α and HIF-2α are not redundant and have specific roles in tumourigenesis, with publications reporting a specific down-regulation of HIF-1α and increased expression of HIF-2α under continuous hypoxia[140,141]. The N-terminus of HIF-α contains bHLH and PAS domains, while the C-terminus of the molecule contains two transactivation domains and one oxygen-dependent degradation (ODD) domain except for HIF-3α **(Figure 2.1)**.

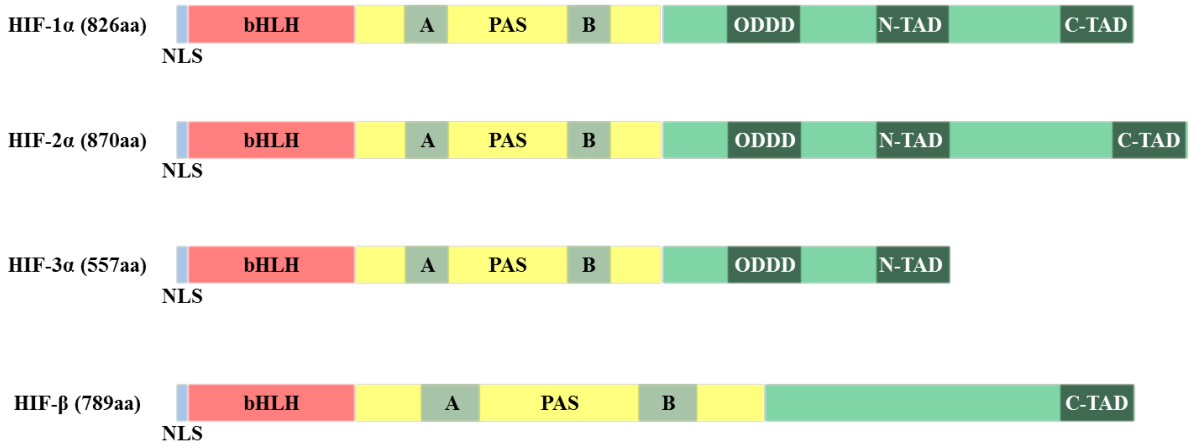

Figure 2.1 Schematic representation of HIF-α and HIF-β structures. The diagram shows the compositions of HIF subunits: HIF-1, 2, 3α and HIF-β. NLS: nuclear localisation signal, bHLH: basic helix loop helix domain, PAS: Per/ARNT/Sim motif, ODDD: oxygen-dependent degradation domain, N-TAD: N-terminal transactivation domain, C-TAD: C-terminal transactivation domain.

The N-terminal bHLH mediates DNA binding and specificity. Both the bHLH and PAS domains mediate heterodimerisation with HIF-β. The ODD domain contains two distinct proline residues (P402 and P564 in HIF-1α), which are the sites for PHD-dependent hydroxylation, thus regulating HIF-α protein stability[142]. HIF-1α and HIF-2α possess both an N-terminal transactivation domain (N-TAD) and a C-terminal transactivation domain (C-TAD), while HIF-3α possess only one transactivation domain[143,144]. Interestingly, the C-TAD also contains an asparagine residue, which is the site of hydroxylation by factor inhibiting HIF (FIH), thus regulating the transcriptional activity of the CAD. Additionally, an inhibitory domain (ID) is located between the two transactivation domains which is necessary for normoxic repression of the CAD.

2.3.2 HIF prolyl-4-hydroxylases (PHDs)

The major regulators of HIF-α are HIF-prolyl-4-hydroxylases (PHDs), which target HIF-α subunits for proteasomal degradation under normoxia through hydroxylation. PHDs, or HIF prolyl hydroxylases (HPHs), are evolutionarily conserved oxygen sensors that use oxygen and 2-oxoglutarate (2-OG) as co-substrates, and iron and ascorbate as co-factors[145]. Three homologs of PHDs, PHD1, 2 and 3, are differentially expressed in mammals: PHD2 is generally the dominant isoform in tissues, with the exception of the testis, in which PHD1 is the most abundant, the heart and skeletal muscle, in which PHD3 expression predominates[146,147]. PHDs have a common structural architecture, with a well conserved C-terminal hydroxylase domain and a more variable N-terminal domain, whose functions are poorly characterised[148]. While PHD1 and PHD2 contain 407 and 426 amino-acid residues respectively, PHD3 is much shorter (239 amino-acid residues) with a much shorter and more divergent N-terminal sequence **(Figure 2.2)**[149].

Figure 2.2 Protein sequence alignment of PHD1, PHD2 and PHD3. PHD1, PHD2 and PHD3 protein sequences are aligned using constraint-based multiple alignment tool (COBALT) from PubMed Central-National Centre for Biotechnology. Red colour represents conserved regions. Numbers in brackets represent the numbers of amino acids within the gaps.

As the most dominant form of PHDs, it is no surprise that prenatal deficiency of PHD2 results in embryonic lethality at E12.5 due to placental and heart defects[150]. Somatic or postnatal ablation of PHD2 results in increased EPO and angiogenesis via HIF stabilisation, and hence in polycythaemia and congestive heart failure[151]. Meanwhile, PHD1-deficient mice are viable with improved hypoxia tolerance in skeletal muscle and liver attributable to reprogramming of anaerobic metabolism[152,153]. Similarly PHD3-knockout mice are viable but with abnormal sympathoadrenal development, demonstrated by a reduction in adrenal medullary secretory capacity, hypo-functional sympathoadrenal system and decreased blood pressure[154]. Fascinatingly, combined genetic ablation of PHD1 and PHD2 leads to increase in haematocrit due to increased EPO expression in the liver of adult mice[146].

2.3.3 HIF pathway

The essential step in the chronic responses to hypoxia is the stabilisation and activation of the HIFs. In the canonical pVHL/PHD/HIF pathway, PHD enzymes hydroxylate two distinct prolyl residues within an ODD domain of HIF-α under normoxia. Hydroxylation of these residues increases the binding affinity of HIF-α to the von Hippel-Lindau (VHL) tumour suppressor protein. VHL acts as a recognition tag, which recruits an ubiquitin E2 ligase complex. This then results in poly-ubiquitination and proteasome-dependent degradation of HIF-α. Under hypoxia, the hydroxylation is reduced and hence the stabilisation of HIF-α increased, allowing it to

form a heterodimer with the constitutively expressed HIF-β. The HIF heterodimer transcriptionally activates a large number of genes involved in the adaptation process to low pO_2 **(Figure 2.3)**[155].

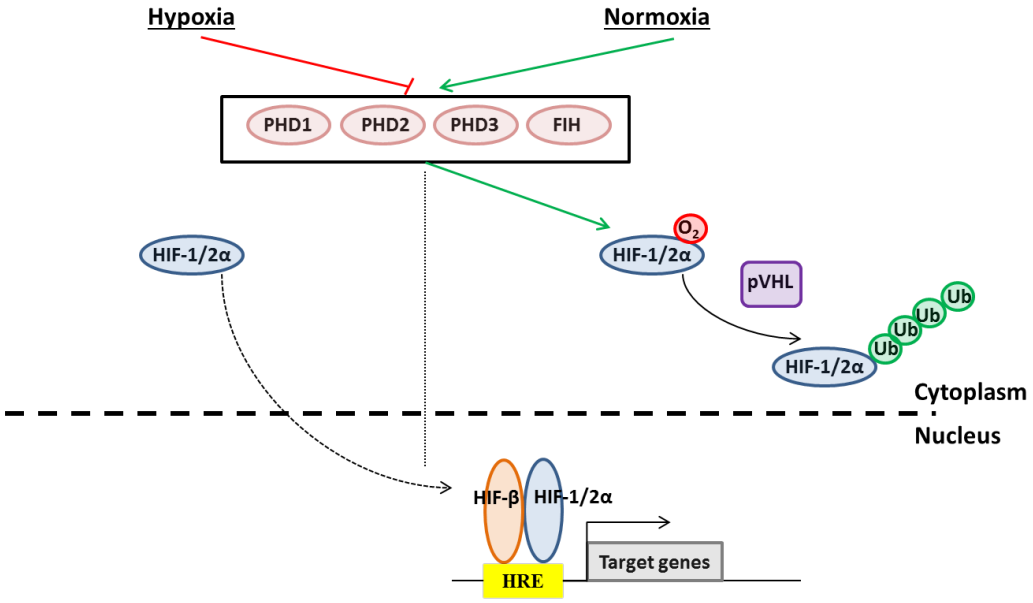

Figure 2.3 Schematic of canonical HIF-signalling pathways. The presence of oxygen resulting in hydroxylation of HIF-α by PHDs and subsequent recognition by pVHL, ubiquitination and proteasomal degradation of the α-subunit. In hypoxia, the hydroxylation does not occur, resulting in stabilisation of HIF-α, dimerization with HIF-β and recognition of the hypoxia-response element (HRE), followed by transcription of target genes.

Despite of the similarity between HIF-1α and HIF-2α, both factors are expressed in distinct cell types[139,156]. HIF-1α expression can be observed in all nucleated cells of metazoan species, while HIF-2α expression is more restricted to specific cell types, such as renal interstitial cells and hepatocytes[156]. Interestingly stabilisation of HIF-1α and HIF-2α under hypoxia displays different kinetics[140,141]. Continuous exposure of a variety of human cancer cell lines to hypoxia results in a down-regulation of HIF-1α whereas HIF-2α protein levels increase[140,141]. This suggests the adaptive activation of HIF depending on exposure to acute or chronic hypoxia and hence regulation of different downstream target genes from HIF-1α or HIF-2α.

2.4 Hypoxia and breast cancer

Hypoxia plays a significant role in tumour development and metastasis[157,158]. Hypoxia is observed in more than half of all cancers and is an adverse prognostic marker in various types of human cancer and is independent of other prognostic factors such as tumour stage, histological grade and lymph node status[159]. The ultimate cause of mortality in breast cancer patients is the propagation of breast cancer cells from the primary tumour site to distant organs in a process termed metastasis[158]. The increased cell proliferation and decreased cell death are hallmarks of breast cancer

cells[160]. An increase in breast cancer cell numbers leads to increased oxygen consumption, hence decreased in O_2 availability (hypoxia). Hypoxia in turn leads to the activation of HIFs and subsequently drives further breast cancer pathogenesis.

The progression of metastasis in breast cancer is a multistep process. The process includes the acquisition of aggressive and invasive phenotypes by the cancer cells, enhancement in the ability to intravasate from the site of origin to the lymphatic or blood vessels and extravasate from the lymphatic or blood vessels into the metastatic site, and finally to ability to revert back to epithelial phenotype for clonal expansion and colonisation of the metastatic site. Moreover, the HIF signalling pathway induces angiogenesis, which is essential for the growth of primary tumour and the progression of metastasis[133]. An example of gene that is hypoxically-regulated is Wnt-1-induced signalling protein-2 (WISP-2)[161].

2.4.1. HIF-2α-mediated regulation of WISP-2

Previously, the dependence of 34 known HIF target genes on HIF-1 and HIF-2 was analysed by our laboratory using quantitative PCR in MCF-7 cells exposed to hypoxia for 4-72 hours and was grouped by K-means clustering into four distinct groups with 8-10 set members **(Figure 2.4)**[161]. Unsurprisingly, the majority of genes investigated were predominantly depending on HIF-1α, such as glycolytic enzymes and genes involved in metabolic adaptation to low oxygen availability, with only a few genes showing activation by HIF-2α. One of the genes selectively activated by HIF-2α is WISP-2, a secreted protein member of the connective tissue growth factor/cysteine-rich 61/ nephroblastoma overexpressed (CCN) family which has previously been reported to be hypoxically induced by cooperation between HIF-2α and the ETS oncogene family member ETS-like gene (ELK-1)[162].

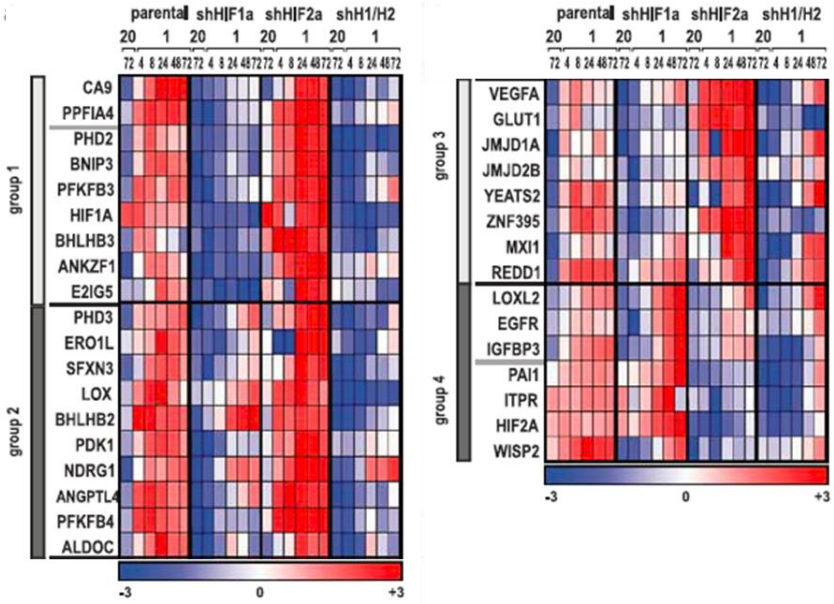

Figure 2.4 HIF target gene clustering based on their distinct hypoxic expression profile. 34 known target genes of HIF were analysed by RT-qPCR and was grouped into four distinct groups (adapted from[161]).

WISP-2, also called CCN5, is a secreted protein member of CCN family and is approximately 29 kDa in size[162]. CCN family proteins consist of 6 members (CCN1-6) which are matricellular proteins with conserved modular domains involved in angiogenesis, cell proliferation, tumourigenesis and wound healing[163-166]. Structurally, except for WISP-2, CCN family proteins contain four conserved domains: insulin-like growth factor binding proteins (IGF-BP), von Willebrand factor-C (VWC), thrombospondin-1 (TSP-1), and cysteine knot (CT) domains **(Figure 2.5)**[167]. The lack of the CT domain in WISP-2 indicates a functional difference compared with other members of the CCN family. In fact, the CT domain has been reported to be a proliferation domain, suggesting a role of WISP-2 in mediating proliferative properties of cells[168].

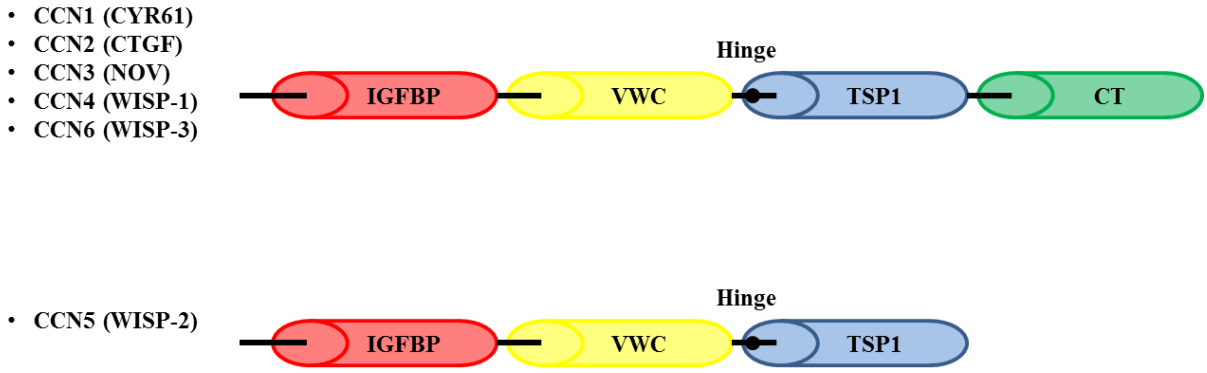

Figure 2.5 Structure of CCN family members. CCN family proteins are composed of 4 motifs separated by a hinge region which is susceptible to proteolytic cleavage. IGFBP: insulin-like growth factor binding protein, VWC: von Willebrand factor-C, TSP1: thrombospondin-1, CT: cysteine knot. (Adapted from [167])

Several publications have asserted the important role WISP-2 in the progression of cancer, including breast cancer, colorectal cancer and pancreatic cancer[169-171]. In breast cancer, WISP-2 is expressed in a biphasic manner, in which it is undetected in normal HMECs, highly expressed in non-invasive breast cancer cells (MCF-7 and T-47D) and again undetected in highly invasive breast cancer cells (MDA-MB-231 and MDA-MB-468)[172]. The loss of WISP-2 in MCF-7 induces estrogen-independent growth and promotes EMT. Re-expression of WISP-2 in highly invasive MDA-MB-231 cells results in reduction of proliferation, which is consistent with a more invasive phenotype[173].

3. Estrogen Signalling

3.1 Introduction

In 1896, British physician George Beatson reported the relationship between cancer of the breast and ovarian function, in which the removal of ovaries caused reduction in circulating estrogen, resulted in remission of advanced breast cancer[174]. His publication heralds the introduction of the significance of hormones in the carcinogenic process[174]. More than 50 years after Beatson's observation, Rous and Kidd in 1941 proposed the multifactorial concept of tumourigenesis and the existence of latent cancer cells based on the studies of skin cancer induction by carcinogens[175]. Further studies led to the generalisation that carcinogenesis is a two-step process, involving initiation and promotion.

It was not until 1975, however, that hormones are acknowledged to be carcinogen-like, not only by a genotoxic mechanism, but also by influencing the rate of cell proliferation and thereby increasing the potential for spontaneous mutations[176]. Furthermore, Jacob Furth proposed that unrepaired copying mistakes during DNA replication could generate cells carrying new karyotypes, which are potential ancestors of novel clones predisposed to become malignant tumours[176]. Furth's description of the carcinogen-like potential of hormones is further supported by MacMahon and colleagues' suggestion that estrogens, generally, and estradiol, specifically, could play a role in human breast cancer carcinogenesis[177].

With the discovery and characterisation of *BRCA1* and *BRCA2*, the importance of inherited traits in breast cancer causation comes to the spotlight[178,179]. Surprisingly however, mutations in these genes contribute little to nothing to the causation of sporadic breast cancer[180]. Moreover, mutations in the *TP53* tumour suppressor gene contribute to increased risk of breast cancer development in certain families, but not generally to the risk of sporadic breast cancer[181]. These observations lead to the current view that under the majority of circumstances, breast epithelial cell does not possess a germline mutation in tumour suppressor genes[181]. Circulating steroid hormones (e.g. ovarian estradiol and to a lesser extent progesterone) drive proliferation of breast epithelium[28,182]. Increasing breast cell proliferation in turn increases the risk of a DNA copying mistake that is not repaired by the DNA repair machineries, which then facilitates expression of genetic errors by loss of heterogeneity that eventually leads to a malignant phenotype[176].

3.2 Steroid hormones

In the human body, generation of steroid hormones occurs *de novo* by biosynthetic pathways in specific endocrine glands (the adrenals and ovaries in women and the adrenals and testes in men) **(Figure 3.1)**[183]. Additionally, steroid hormones can be produced in peripheral tissues from circulating precursors that are generated by the endocrine glands. Important peripheral tissues include the liver, kidney, breast, prostate, brain and skin.

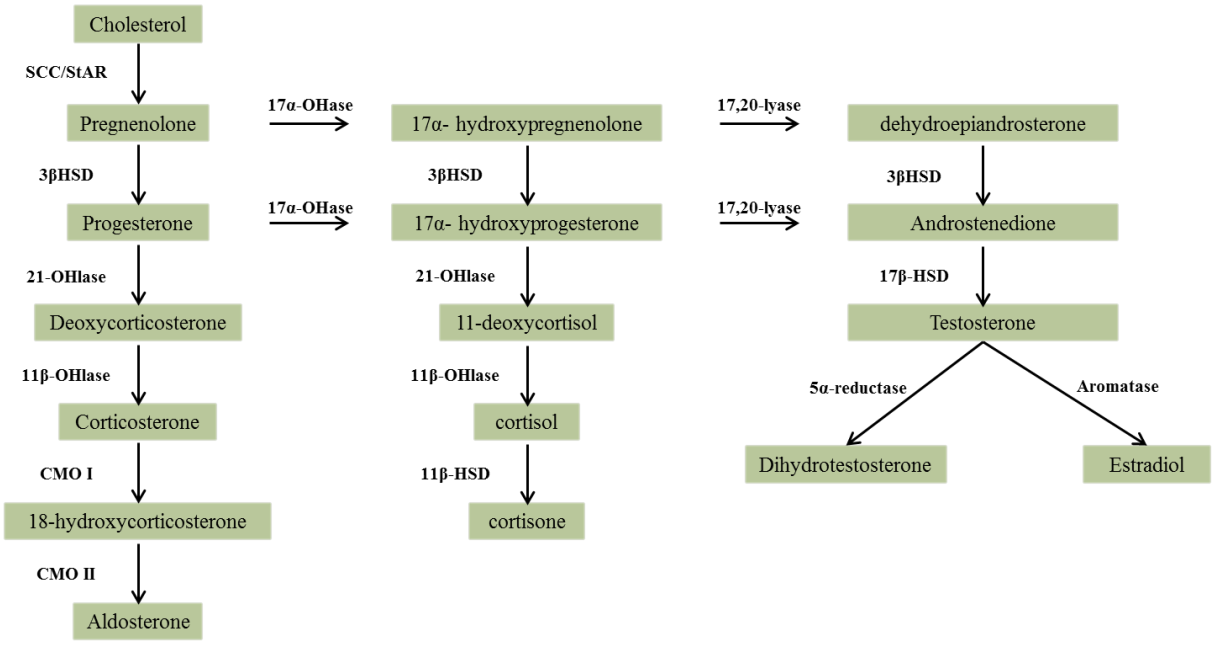

Figure 3.1 Biosynthesis of steroid hormones from cholesterol. Cholesterol serves as the precursor to the generation of progesterone, aldosterone, cortisone, estrogen and testosterone (Adapted from [35]).

The first step in the biosynthesis of steroid hormones in the endocrine glands is the conversion of cholesterol to pregnenolone[184]. The conversion reaction occurs in the mitochondrion and is catalysed by the cholesterol side-chain cleavage cytochrome P-450 (P450scc) enzyme together with auxiliary electron-transferring proteins[184]. Following the conversion of cholesterol to pregnenolone by the mitochondrial side-chain cleavage system, pregnenolone can be converted to either progesterone or 17α-hydroxypregnenolone, which is catalysed by 3β-hydroxysteroid dehydrogenase (3β-HSD)[9].

Pregnenolone and progesterone can undergo hydroxylation at carbon 17, and subsequent cleavage of the side chain at carbon 17 catalysed by P450c17, generating dehydroepiandrosterone (DHEA) and androstenedione respectively[185]. 5-Androsterone-3β, 17β-diol and testosterone are converted from DHEA and androstenedione, respectively, through the action of 17β-hydroxysteroid dehydrogenase (17β-HSD)[186]. Lastly, testosterone is converted into estradiol through the action of a complex microsomal aromatase cytochrome P-450 (P450arom) in reactions called aromatisation[9].

3.3 Estrogen

Estrogens are the primary female sex hormones, which have been shown to regulate diverse physiological processes in both reproductive and non-reproductive tissues, such as in the mammary, cardiovascular, hepatic and osseous tissues[187-189]. In human, estrogen consists of 17β-estradiol (E2), the most abundant and potent estrogen, and its less potent metabolites estrone (E1) and estriol (E3) **(Figure 3.2)**[190]. E2 is the major estrogen during the pre-menopausal period of a female's life, while E1 plays a larger role after menopause, when it is generated from adrenal DHEA in adipose tissue[191]. E3, which is converted from E1 through 16α-hydroxylation, is generated by the placenta during pregnancy[191].

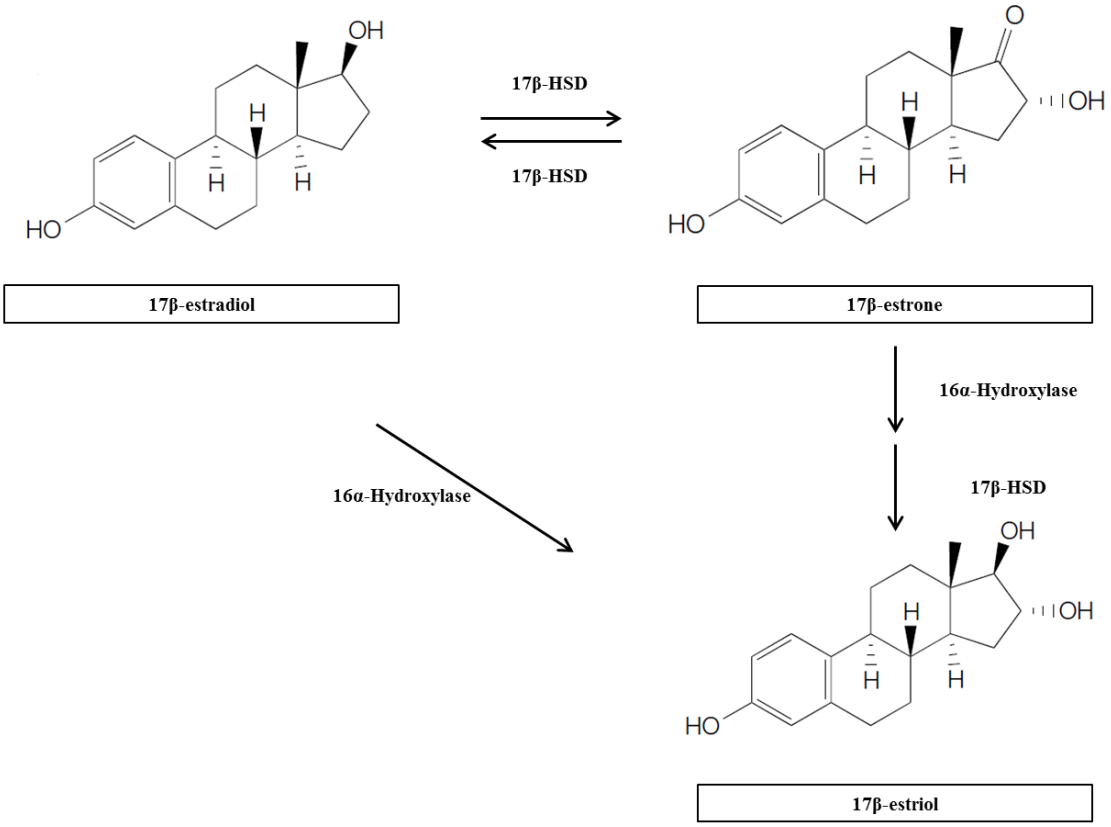

Figure 3.2 Structures of estrogens. Three major forms of estrogens are present in females: 17β-estrone (E1), 17β-estradiol (E2) and 17β-estriol (E3)[190].

3.3.1 Biosynthesis of estrogen

In pre-menopausal women, the major sites of estrogens synthesis are the corpus leteum of the ovaries and the placenta, with a small but substantial amount of estrogens also generated by non-gonadal organs, such as the liver, heart, skin and brain[191,192]. The estrogens produced in the ovaries of pre-menopausal women are directly released into the bloodstream, where they act on their corresponding receptors in the target organs. In non-reproductive women, such as pre-puberty females and post-menopausal females, non-gonadal organs are the major sources of estrogens[191]. The non-gonadal organs include kidney, adipose tissue, skin and brain[191,192]. However, unlike the bloodstream-bound estrogens produced in the ovaries, non-gonadal organ-synthesised estrogens act mostly within the vicinity of the site of synthesis in a paracrine or intracrine manner[192]. Importantly, estrogens are also synthesised in male testes and are essential in the regulation of normal male gonadal development and spermatogenesis[193].

In a healthy reproductive age woman, estrogen synthesis in the ovaries occurs during the process of folliculogenesis, the maturation of the ovarian follicle **(Figure 3.3a)**[194]. Two major cell types within the ovaries are involved in the synthesis of estrogen in a cooperative manner: the Thecal cells and Granulosa cells[35]. The complementarity of these two cells can be observed by the sharing of burdens, in which the thecal cells provide both structural support to the follicle and being the sole producer of androgens from progesterones, which are required for estrogen synthesis in the neighbouring granulosa cells[194]. In folliculogenesis, the activation of the primordial follicle induces the formation of secondary follicle and simultaneously signals the stroma surrounding the follicle to recruit thecal cells[194]. The recruited thecal cells surround the follicle and start to differentiate. Upon the release of luteinising hormone (LH) from the pituitary, androgens are synthesised by the necessary enzymes located within the mitochondria and endoplasmic reticulum of the thecal cells[194]. Subsequently, the synthesised androgens are transported to the granulosa cells, where they are converted into E1 and E2 by P450arom **(Figure 3.3b)**[185].

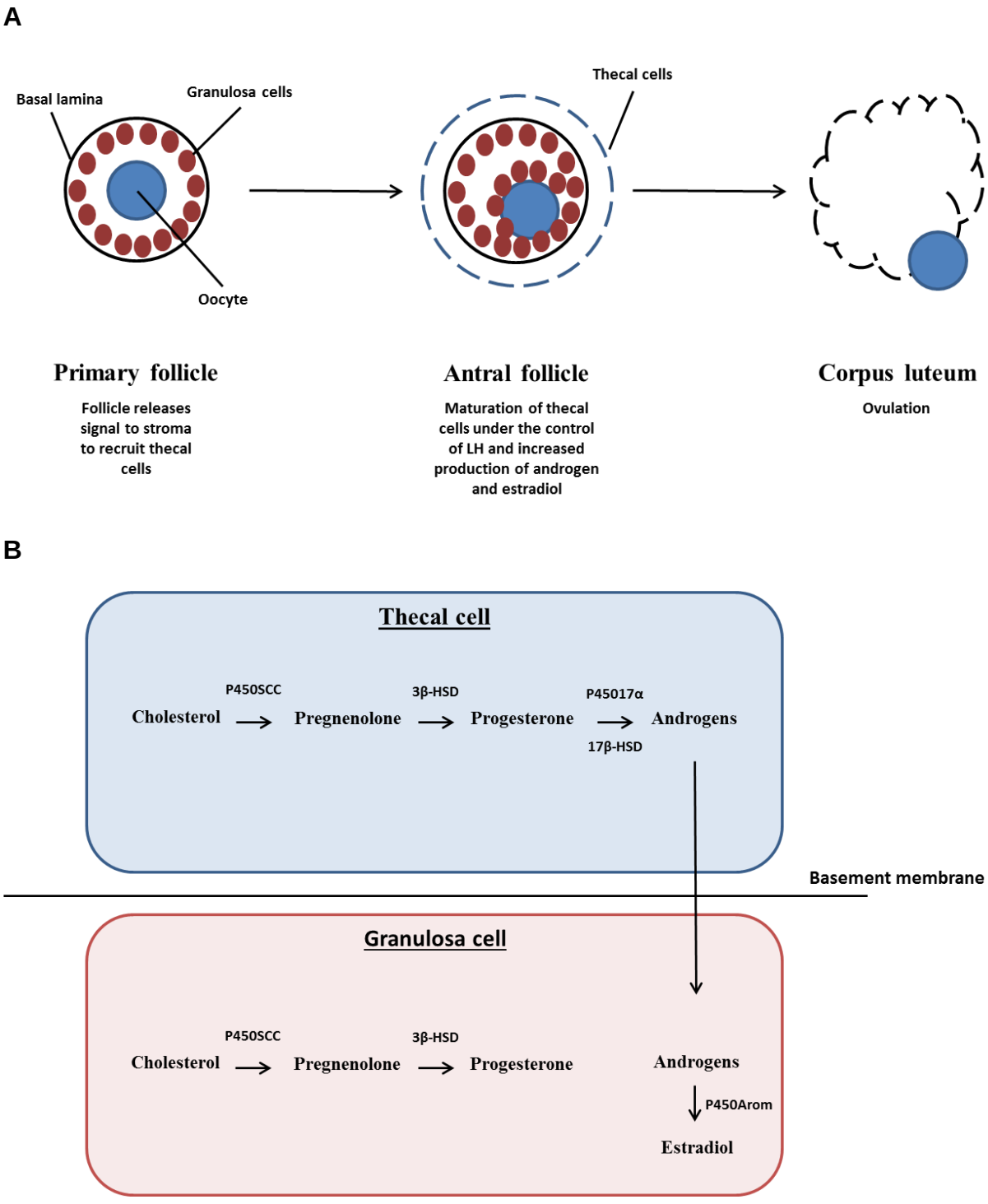

Figure 3.3 Biosynthesis of estrogen in the ovaries. (A) The stages of follicle development during folliculogenesis (Adapted from [35]). **(B)** Estrogen synthesis in the ovaries depends on the cooperation between thecal and granulosa cells (adapted from [194]).

3.3.2 Estrogen receptors

The effects of estrogens are mediated primarily via their receptors, estrogen receptors (ERs), which include the classical receptors such as ERα and ERβ, and membrane-bound receptors such as G protein-coupled estrogen receptor 1 (GPER) and ER-X[195-197]. Although, both types of ER are activated by estrogen and facilitate downstream effects, the nuclear ERs mediate transcriptional changes in a time course of hours or days, while the membrane-bound ERs trigger a rapid intracellular signalling cascade. ERα and ERβ are members of the nuclear receptor (NR) superfamily that function as ligand-inducible transcription factors. ERα and ERβ are encoded by different genes located on different chromosomes, *ESR1* on chromosome 2 and *ESR2* on chromosome 14 respectively. Both ERα and ERβ possess similar architecture, consisting of six modular domains that are functionally distinct: domain A-F **(Figure 3.4)**[198].

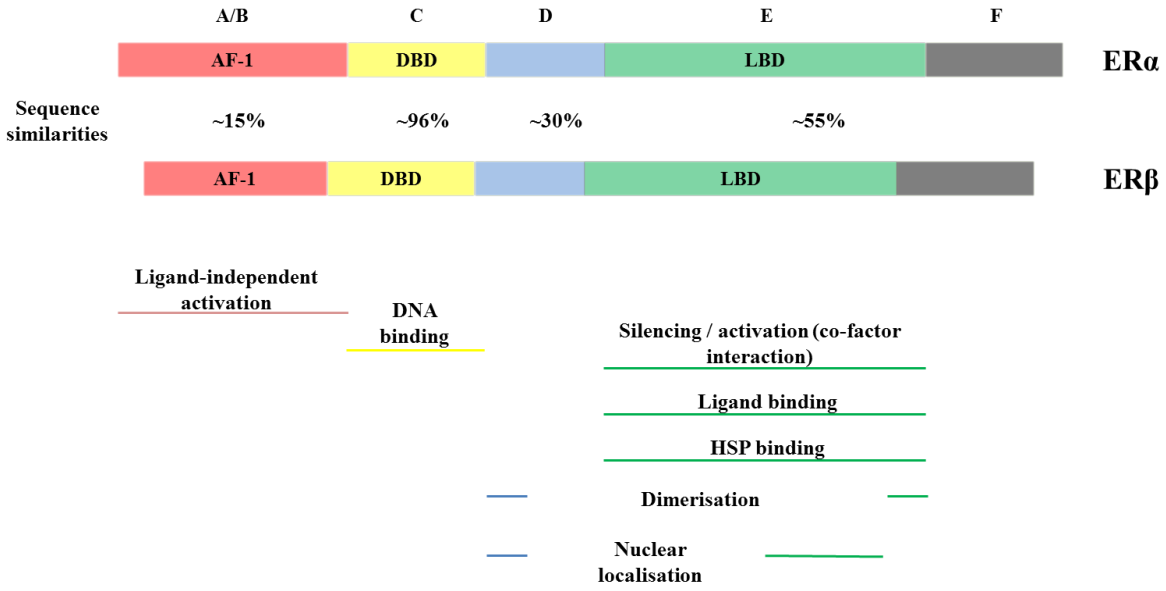

Figure 3.4 Structures of ERα and ERβ. ER consists of ERα and ERβ, which possess striking similarities (96% sequence homology) in their DNA binding domain (DBD). However they are functionally distinct, due to differences in the activation domains (AF-1 and AF-2)[198].

The A/B domain of ERs is highly variable (approximately 15% sequence similarity) and contains a ligand-independent trans-activation function (AF-1)[198]. The C functional domain contains a highly conserved DNA binding domain (DBD) which targets ER to their cognate binding sites containing estrogen response elements (EREs, 5'-GGTCANNNTGACC-3')[199]. Nuclear localisation sequence (NLS) and dimerisation region are located within the C functional domain. The D domain or the hinge domain is the most variable region among NRs and represents a linker between the DBD and the ligand binding domain (LBD) and it also plays a role in the stabilisation of the LBD[198]. The LBD or the E domain specifically binds with estrogens[198]. Moreover, this domain also possesses dimerisation, co-factor interaction, heat shock protein (hsp) binding, nuclear localisation regions and the ligand-dependent activation function (AF-2). The AF-2 has been reported to be

implicated in transcriptional regulation via the recruitment of the co-regulators[200,201]. Finally, the carboxyl-terminal F domain is highly divergent and is involved in transcriptional modulation and receptor dimerisation[202,203].

3.3.3 Estrogen signalling pathways

Estrogen signalling pathways can be broadly classified into four different mechanisms based on the dependence on ER, the initiation sites, and the ligand requirement[191]. Although most estrogen-mediated signalling pathways are ER-dependent, ER-independent signalling pathways occur. ER-dependent pathways can further be classified based on whether the initiation occurs in the nucleus or plasma membrane. Furthermore, an ER-independent signalling pathway has been reported recently in the improvement of vascular function by estrogen[204].

The classical mechanism is an ER-dependent, nuclear-initiated estrogen signalling pathway **(Figure 3.5)**. Estrogen binding triggers the dissociation of hsp90 from the ER, followed by dimerisation and nuclear translocation of the ligand-receptor complex. The activated ERs can form either homodimers or heterodimers, which regulate distinct sets of E2-responsive genes[205]. Mutation analysis indicated that the dimerisation interface required in the formation of heterodimerisation is very similar to that required for homodimerisation[206]. Additionally, the ERα/ERβ heterodimers are capable of interacting with co-regulators[206]. These observations suggest that the presence and the ratio of ERα and ERβ within different cell types determine the regulation of E2-responsive genes. Inside the nucleus, the estrogen/ER dimeric complex recruits the transcriptional machinery and other co-factors and binds to the EREs of the target genes and induces or represses target gene transcription. Besides binding directly to the EREs, activated ERs can form an indirect association with the DNA by interacting with other transcription factors, such as stimulating protein-1 (SP-1) and activator protein-1 (AP-1)[207,208].

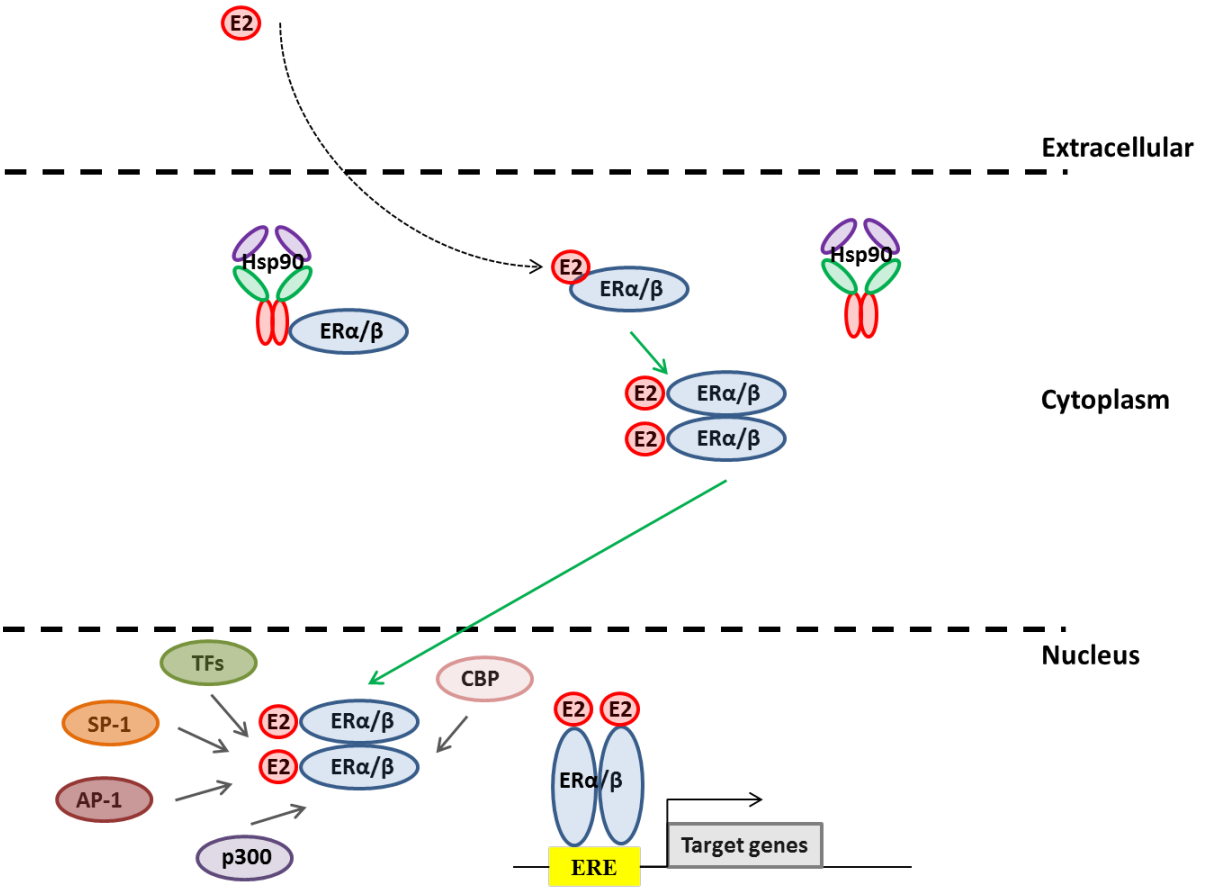

Figure 3.5 Schematic representation of the classical ER-dependent nuclear-initiated estrogen signalling pathway. The binding of estrogen to ERα/β induces dimerization of the receptors and translocation to the nucleus, where the activated receptor dimer interacts with other transcription factors to induce transcription of downstream target genes.

An alternative to the nuclear-initiated pathway is the ER-dependent, membrane-initiated estrogen signalling. Unlike the longer time it takes for the nuclear-initiated pathway, the membrane-initiated estrogen signalling is usually acting rapidly either via crosstalk with other membrane receptors or via activation of a variety of cytoplasmic signalling cascades **(Figure 3.6)**[190]. ERα and ERβ have been reported to mediate downstream signalling through the activation of a variety of protein kinase cascades, such as MAPK/ERK and PI3K/AKT pathways[190]. In addition to membrane-bound ERα and ERβ, rapid signalling by estrogen is also mediated by GPER and ER-X[197,209]. GPER is an estrogen-induced membrane receptor and is located in the plasma membrane, Golgi apparatus, and endoplasmic reticulum[210]. GPER has been reported to mediate rapid estrogen signalling via its association with the phosphorylation of ERK1/2, activation of PI3K and PLC, regulation of intracellular cAMP and Ca^{2+} levels[209,211-215]. ER-X, a high-affinity, saturable membrane-bound ER, is expressed in the brain, uterus, and lungs, and has been reported to be functionally distinct from ERα and ERβ[197].

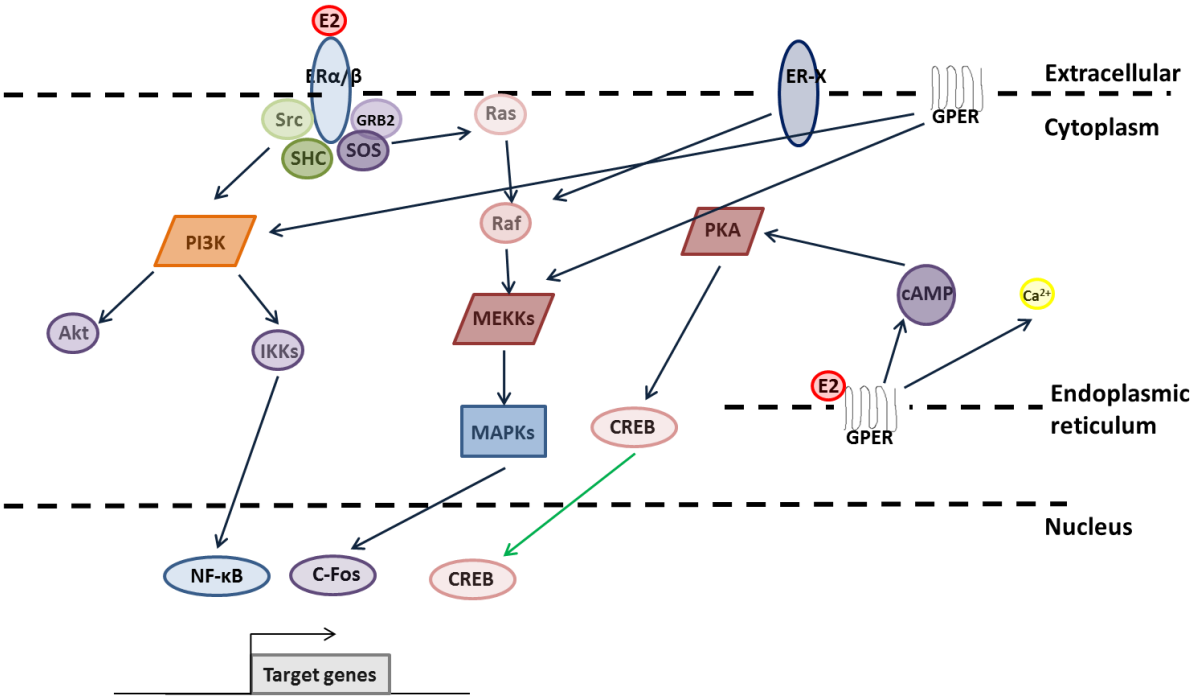

Figure 3.6 ER-dependent, membrane-initiated estrogen signalling pathway. The membrane-initiated estrogen signalling is usually rapid and involves a variety of protein kinase cascades.

Recently more studies have shown estrogen signalling independent of the activation of ERs[216-218]. The phenolic A ring of estrogen possesses an intrinsic anti-oxidant property, which provides estrogen with the ability to exert anti-oxidant effects and suppress oxidative stress by hydrogen peroxide, superoxide anions and other pro-oxidants in an ER-independent manner[219-221]. Furthermore knockout experiments have shown that E2 treatment can promote breast cancer development in mice lacking ERα and ERβ expression, suggesting ER-independent effects on tumour formation[222]. Moreover, further studies have reported the presence of ligand-independent estrogen signalling pathways[216-218]. In this pathway, a variety of factors, such as the neurotransmitter dopamine, the growth factors epithelial growth factor (EGF) and insulin-like growth factor (IGF) and the protein kinases PKC, PKA, MAPK, PI3K, can activate ERs mostly via phosphorylation[216,217,223-226].

3.4 Hypoxia and estrogen signalling in breast cancer

Besides regulating numerous aspects of human physiology, estrogens have also been reported to influence diverse pathophysiological processes, such as carcinogenesis, cardiovascular diseases, osteoporosis and Alzheimer's disease[227-230]. Prolonged exposure to E2 is one of the major factors in breast carcinogenesis[231]. ERs, especially ERα, exert a major role in the development of breast cancer; therefore providing the rationale behind the use of tamoxifen, an ER antagonist, as a primary treatment option for most breast cancer patients[99]. The importance of ERα in breast cancer development is further supported by the overexpression of ERα in approximately 70% of breast cancer diagnosed[96,231]. Another hallmark of cancer cells

in general and breast cancer cells specifically is their ability to adapt to various changes in oxygen supply. Oxygen partial pressures differ substantially in solid tumours, ranging from normal oxygen partial pressure (normoxia) condition near the circulation to complete oxygen deprivation (anoxia) in the core of the tumour. One of a few factors promoting the survival of breast cancer cells depends on the adaptability of the cells to hypoxia, which is mediated by HIFs. With the significance of both hypoxia and estrogen in breast cancer development, surprisingly little attention has been paid on the interaction between these two contrasting signalling pathways.

3.4.1 Estrogen-dependent regulation of HIF-1α

E2 has been reported to attenuate the hypoxic induction of HIF-1α and subsequent attenuation in EPO expression in Hep3B cells via ER-dependent protein regulation[232]. Interestingly, exposure of MCF-7 breast cancer cell lines to E2 results in rapid induction of HIF-1α protein synthesis, with expression of HIF-1α under normoxia observed by immunoblot experiments[233]. The increase in HIF-1α protein synthesis occurs due to the activation of PI3K/AKT pathway by the E2-ERα complex, leading to the phosphorylation and activation of mammalian target of rapamycin (mTOR) protein[233]. Subsequently, mTOR stimulates protein translation, including HIF-1α protein translation, by phosphorylation of p70 S6 kinase and 4EB-P[233]. The opposing results of HIF-1α regulation by estrogen might be due to the different factors expressed in the two different cell lines.

Contrary to HIF-1α activation by ERα, ERβ has been reported to play an opposing role[234]. ERβ has been predicted to play an essential role in prevention of breast cancer development and metastasis, with reports indicating the inhibitory effects of ERβ in proliferative activity *in vitro*[235,236]. Furthermore, ectopic overexpression of ERβ significantly reduced tumour growth in severe combined immunodeficiency (SCID) mice[235]. Consistent with the opposite effects of ERβ to ERα, transcriptional activity of HIF-1α is inhibited by ERβ, which is mediated via ubiquitin-dependent degradation of HIF-β with subsequent reduction of HIF-1α/HIF-β complex and hence reduction in DNA binding and thus transcriptional activity[237].

3.4.2 Estrogen-dependent regulation of HIF-2α

The potential involvement of E2 in regulating HIF-1α has been previously reported as described above. Nonetheless, there is currently no report regarding the effects of E2 exposure on the regulation of HIF-2α. HIF-2α has been reported to be a positive prognostic factor in breast cancer, which has been further confirmed with the designation of HIF-2α in a list of genes associated with favourable outcome based on studies with different cohorts of breast cancer patients[161,230]. Combined with various observations of HIF-1α regulation by E2, we aimed for the investigation of the potential connection between hypoxia and estrogen signalling in breast cancer, and in particular to the estrogen signalling-dependent regulation of HIF-2α.

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4. Aims of the thesis

The aims of the thesis are to:

1. Examine the role of HIF-2α in breast tumourigenesis by elucidating the regulation of WISP-2 by HIF-2α and to explore the roles of both HIF-2α and WISP-2 in the aggressiveness of breast cancer, and hence their contribution in overall survival in breast cancer patients.
2. Investigate the potential regulation of HIF-2α by estrogen signalling in breast cancer cell lines.

5. Manuscript I: HIF mediated induction of WISP-2 contributes to attenuated breast cancer progression

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5.1 Abstract

Hypoxia and the hypoxia-inducible factor (HIF) signalling pathway trigger the expression of several genes involved in cancer progression and therapy resistance. Transcriptionally active HIF-1 and HIF-2 regulate overlapping sets of target genes and only few HIF-2 specific target genes are known so far. Here we investigated the oxygen-regulated expression of the Wnt-1 induced signalling protein 2 (WISP-2) which has been reported to attenuate breast cancer progression. WISP-2 was hypoxically induced in low-invasive luminal-like breast cancer cell lines on both mRNA and protein levels, mainly in a HIF-2α dependent manner. HIF-2 driven regulation of the WISP-2 promoter in breast cancer cells is almost entirely mediated by two, phylogenetically only partially conserved, functional hypoxia response elements (HREs) located in a microsatellite region upstream of the transcriptional start site. High WISP-2 tumour levels were associated with increased HIF-2α, decreased tumour macrophage density and a better prognosis. Silencing WISP-2 increased the anchorage-independent colony formation and recovery from scratches in confluent cell layers of normally low-invasive MCF-7 cancer cells. Interestingly, these changes in cancer cell aggressiveness could be phenocopied by HIF-2α silencing, suggesting that the direct HIF-2 mediated transcriptional induction of WISP-2 gene expression might at least partially explain the association of high HIF-2α tumour levels with prolonged overall survival of breast cancer patients.

Keywords: invasion, metastasis, motility, oxygen, tumour, transcriptional regulation

5.2 Introduction

Temporally and spatially variable tissue hypoxia is characteristic of solid tumors.[1] Hypoxia-inducible factors (HIFs) allow cancer cells to adapt to microenvironmental tissue hypoxia, affecting all aspects of tumour progression, including metabolism, proliferation, inflammation, angiogenesis and metastasis.[2-5] Importantly, HIFs are also involved in cancer therapy resistance and overall survival correlates with HIFα levels in a cancer type-specific manner.[6-8] Transcriptionally active HIFs are heterodimers usually composed of a constitutively expressed β subunit and either a HIF-1α or a HIF-2α subunit, whose stability and activity is regulated by oxygen-dependent protein hydroxylation.[9, 10] Despite their high structural similarity and identical DNA sequence recognition, several studies identified specific roles of HIF-1 and HIF-2 in tumorigenesis.[11-13] We and others previously showed in a variety of cancer cell lines that HIF-1α protein levels decreased under prolonged hypoxia while HIF-2α levels increased, suggesting a HIFα isoform-specific kinetics of target gene expression.[8, 14, 15] In contrast to the many known HIF-1 and HIF-1/HIF-2 target genes,[8, 14, 16] only few genes have been reported to be regulated exclusively by HIF-2, including erythropoietin, ephrin A1, VE-cadherin, protein tyrosine phosphatase receptor-type Z polypeptide 1, amphiregulin, and Wnt-1 induced signalling protein 2 (WISP-2).[8, 14, 17-21]

WISP-2 is a secreted protein member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family, and is also known as CCN5.[22, 23] WISP-2 has been detected in adult skeletal muscle, colon, ovary and fetal lung, as well as in the stroma of breast tumours derived from Wnt-1 transgenic animals.[24] WISP-2 expression has been shown, in most studies, to inversely correlate with the aggressiveness of breast, pancreatic and colon cancer, suggesting a tumour suppressor-like activity.[25, 26] WISP-2 shows transiently elevated levels during breast cancer progression: while it is almost undetectable in normal human mammary epithelial cells, it is highly expressed in estrogen receptor (ER) positive non-invasive breast cancer cell lines (including MCF-7, BT-474, ZR-75-1, T-47D), and again undetectable in ER negative highly invasive cells (including MDA-MB-231, MDA-MB-468, BT-20 and DU-4475).[27-30] Loss of WISP-2 in MCF-7 cells induced an estrogen-independent growth and promoted epithelial-to-mesenchymal transition, consistent with a more invasive phenotype, whereas forced WISP-2 expression in MDA-MB-231 cells reduced proliferation and invasiveness.[30] WISP-2 expression is induced by estrogens as well as by epidermal growth factor (EGF) and insulin-like growth factor (IGF-1) in ER-positive cells, and WISP-2 is necessary for estrogen and IGF-1 induced proliferation.[31-35]

A functional estrogen response element (ERE) has been identified in the WISP-2 promoter which is required for the estrogen inducibility of the WISP-2 gene.[29] ERα recruits the histone acetyl transferase CREB-binding protein (CBP) as well as the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ to the WISP-2 promoter, suggesting a cooperative control of WISP-2 gene expression.[29, 36] Hypoxia has been identified as

another stimulus of WISP-2 gene expression which is mediated specifically by HIF-2α in cooperation with the ETS oncogene family member ELK-1 in MCF-7 cells.[14, 37] We previously demonstrated that the WISP-2 promoter is induced specifically by HIF-2α in MCF-7 cells.[8] Here, we identified the hypoxia response elements (HREs) and characterized a microsatellite region responsible for the HIF-2α-specific induction of the WISP-2 promoter. Furthermore, we assessed the impact of hypoxia and HIF-2α on WISP-2 mediated cell proliferation, clonogenic growth and motility.

5.3 Material and methods

Cell culture and transfection

Human breast cancer cell lines MCF-7, T-47D, ZR-75-1, BT-474, MDA-MB-468 and MDA-MB-231 were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA). Hypoxic experiments were performed at 1% oxygen and 5% CO_2 in a gas-controlled glove box (InvivO2 400; Ruskinn Technology Ltd, Bridgend, United Kingdom) as described previously.[38] Viral particles were generated in HEK293T cells following co-transfection of the transfer vector (3 μg) together with the packaging plasmids pLP1 (4.2 μg), pLP2 (2 μg) and pVSV-G (2.8 μg) (Invitrogen, Carlsbad, CA, USA) using polyethylenimine (Polysciences, Warrington, PA, USA). MCF-7 cells were infected with viral particles and cell pools were selected with puromycin as described before.[38] Two independent WISP-2 shRNA vectors (TRCN0000033355 and TRCN0000033357) were obtained from Sigma-Aldrich. shRNA resistant HIF-2 α was used to rescue MCF-7 cells with stable HIF-2α knock-down as described before.[8]

mRNA and protein detection

Total cellular RNA was extracted as previously described.[39] Total RNA (2 μg) was reverse transcribed (RT) using AffinityScript reverse transcriptase (Agilent, Santa Clara, CA, USA) and the cDNA levels were estimated by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in a MX3000P light cycler (Agilent). Transcript levels were calculated by comparison with a calibrated standard and expressed as ratios relative to ribosomal protein L28 mRNA levels. Immunoblots were performed as previously described.[40] Antibodies against the following proteins were used: WISP-2 (Abcam, Cambridge, United Kingdom), HIF-1 α (BD Transduction Laboratories, Allschwil, Switzerland), HIF-2 α (Novus Biologicals, Littleton, CO, USA), Sp1 (Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (Sigma-Aldrich). Breast cancer tissue microarray analysis has been described previously.[8]

Plasmid construction and reporter gene assays

The promoter truncations -1919/+16, -808/+16, -520/+16, and -422/+16 have been reported previously[29] and were kindly provided by M. Sabbah (Paris, France); -422/-75, -83/+16 and MS deletions were generated by standard cloning techniques. The

WRE1, CRE, ELK-1 and WRE2 sites in the -422/+16 construct were mutated by site-directed mutagenesis. The -252/+16 truncation was generated by PCR cloning. The -422 $(CA)_n$ promoter region was amplified from T47D genomic DNA, transformed into bacteria, and isolated for sequencing to determine the CA repeat length. The -112/+16 and the -112/+16 HRE2/3 double-mutant constructs were generated by elongation of an internal MluI site using double-stranded oligonucleotides. HRE2 and HRE3 in the -422/+16 construct were mutated by site-directed mutagenesis (5'-CGTG-3' to 5'-CAAA-3'). Dual luciferase reporter gene assays were performed as described previously.[41]

Genomic DNA extraction and sequencing

Cells were washed with PBS, re-suspended in high salt lysis buffer (200 mM NaCl, 50 mM Tris/HCl pH 8.0, 50 mM EDTA pH 8.0, 1% SDS) and treated overnight with proteinase K (20 mg/ml). After addition of saturated NaCl, DNA was precipitated with isopropanol, washed with 70% ethanol and re-suspended in Tris/EDTA buffer. WISP-2 genomic DNA was amplified by PCR using forward primer 5'-tacgggtaccacggacaggcacccccttggtgg-3' and reverse primer 5'-cagatgtgcagagccagcagctt-3'. PCR products were gel-purified and sequenced (Microsynth, Balgach, Switzerland).

Cellular proliferation, colony formation and motility assays

To determine cell proliferation and viability, 10^5 cells per well were seeded into 6-well plates, allowed to adhere overnight, exposed to normoxia or hypoxia for 0 to 72 hours, detached by trypsin/EDTA and counted using a Beckman Coulter Vi-cell ™ XR Cell Viability Analyser (Brea, CA, USA). For low cell density colony forming assays, $2x10^3$ cells per well were plated into 6-well plates, allowed to adhere overnight, and exposed to normoxia or hypoxia for 10 days with the medium replaced every 3 days. The colonies were fixed with methanol, stained with 0.5% crystal violet and counted. For anchorage independent colony formation assays, 10^4 cells were re-suspended in 2 ml 0.4% low melting agarose (Sigma-Aldrich) in DMEM, poured on top of a 2% low melting agarose layer in 6-well plates and allowed to settle overnight. Following exposure to normoxia or hypoxia for 14 days, the soft agar was washed with PBS, the colonies stained with 0.005% crystal violet in methanol for 1 hour at room temperature, and counted. For scratch assay, cells were allowed to grow to 100% confluency in 12-well plates. Following crosswise scratching with a 200 µl pipette tip, the cells were exposed to normoxia or hypoxia in FCS-free DMEM for 24 hours. The cell-free area was measured and converted to % recovery.

Statistical analyses

If not indicated otherwise, unpaired Student's t-tests were applied. Differences between two values were considered statistical significant if $p < 0.05$. Asterisks shown in the figures indicate the following levels of significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

5.4 Results

The HIFα isoform responsible for hypoxic WISP-2 induction is specific for distinct breast cancer cell lines

To determine the HIF α isoform directing WISP-2 expression in breast cancer, four luminal-like (MCF-7, BT-474, T-47D and ZR-75-1), one basal A-like (MDA-MB-468) and one basal B-like (MDA-MB-231) human breast cancer cell line were analyzed. Similar to data published previously,[8, 30] constitutive WISP-2 mRNA levels were high in MCF-7 cells, moderate in T-47D and BT-474 cells, low in ZR-75-1 and almost undetectable in MDA-MB-231 and MDA-MB-468 (Figure 1A). Although WISP-2 immunoblotting is notoriously difficult,[42] probably because the WISP-2 protein is partially secreted, at least in MCF-7 cells also WISP-2 protein could be detected and was found to be upregulated following hypoxic stimulation (Figure 1B), like previously published for the WISP-2 mRNA levels.[8]

Immunoblot analyses revealed that all six cell lines express both HIF-1 α and HIF-2 α in a hypoxia-inducible manner (Figure 1C). The variable relative molecular weight of HIF-1 α between approx. 98 to 120 kDa, as observed in the different cell lines, is due to varying degrees of phosphorylation like reported previously.[43] HIF-1 α and HIF-2 α were stably knocked-down by viral transduction of shRNA constructs. Exogenous shRNA expression efficiently reduced HIF α levels (Figure 1C). In the absence of HIF-1 α increased HIF-2 α protein levels could be observed in most cell lines, a currently unexplained phenomenon that we and others described previously.[8, 44]

In addition to MCF-7 cells[8], a robust hypoxic WISP-2 mRNA induction was also found in T-47D, BT-474 and ZR-75-1 cells (Figure 1D). No hypoxic WISP-2 induction could be detected in MDA-MB-231 and MDA-MB-468 cells (data not shown). The knock-down of the HIF α isoforms demonstrated that WISP-2 mRNA is predominantly regulated by HIF-2 α in MCF-7 and T-47D cells (Figure 1D). In MCF-7 cells, HIF-2 α shRNA also prevented the hypoxic induction of WISP-2 but not of prolyl-4-hydroxylase domain 2 (PHD2) protein levels, the latter being a HIF-1 α target (Figure 1B). However, in BT-474 cells hypoxic WISP-2 mRNA induction was HIF-1 α but not HIF-2 α dependent, and in ZR-75-1 cells hypoxic WISP-2 mRNA induction was attenuated in the absence of both HIF-1 α and HIF-2 α (Figure 1D). These data demonstrate that WISP-2 is regulated by hypoxia in low-invasive luminal-like breast cancer cell lines and that cancer-specific HIF α isoforms confer hypoxic WISP-2 induction.

Potential enhancer elements involved in HIF-inducible WISP-2 promoter activity

To further investigate HIF α isoform specific effect on WISP-2 transcription, we tested various WISP-2 promoter truncations driving firefly luciferase reporter gene expression in MCF-7 cells. All constructs ranging from -1929/+16 to -422/+16 relative to the transcriptional start site (TSS) of the human *WISP2* gene displayed similar promoter activities. In contrast to the robust hypoxic WISP-2 mRNA induction (Figure

1C), only weak responses to hypoxia could be observed with these reporter gene constructs (Figure 2A). Whereas moderate increases in promoter activity, mainly under hypoxic conditions, were observed after HIF-1 α overexpression, strong increases in normoxic as well as hypoxic promoter activities followed HIF-2 α overexpression, suggesting that HREs likely are present on these constructs but are not sufficient to confer full hypoxic inducibility. Additional truncations of the -422/+16 construct resulted in the complete loss of promoter activity in the absence of the region close to the TATA-like box (construct -422/-75), demonstrating that the first 75 bp are essential for basal promoter activity. Normal basal activity but unresponsiveness to HIF α overexpression was observed for construct -83/+16 (Figure 2B).

Three single putative HREs (HRE1, HRE2 and HRE3) and a double HRE (HRE4) were identified in the *WISP2* promoter region (Figure 2C). Of note, the three single HREs were all located within microsatellite (MS) regions. HRE1 was found in a CA repeat region denominated MS I, HRE2 was located at the transition between a GT and a GC repeat, collectively named MS II, and HRE3 was identified within the GC repeat of MS II (Figure 2C). Furthermore, a phylogenetic footprint analysis of the promoter region revealed a strong sequence conservation around the TATA-like box, comprising a potential cAMP response element (CRE), a binding site for the Ets-like gene 1 (ELK-1), and two WNT responsive elements (WREs) (Figure 2C). However, when tested in MCF-7 shH2a cells with suppressed endogenous HIF-2 α levels, WRE1 mutation neither affected basal promoter activity nor HIF α responsiveness. Mutation of the CRE site strongly decreased promoter activity but partially retained HIF-2 α -mediated induction. Similarly, the ELK and WRE2 mutations reduced overall promoter activity and slightly affected the HIF-2 α -responsiveness (Figure 2D). In conclusion, these conserved elements are mainly required for basal promoter activity but do not seem to confer the HIF-2 α -dependent induction of the *WISP2* promoter, although we cannot exclude a partial cooperation between these *cis*-acting elements with the HREs.

Two HREs within a microsatellite repeat regulate HIF-dependent WISP-2 transcription

To identify the HRE(s) responsible for HIF-mediated WISP-2 regulation, MS I (containing HRE1) was removed by a -252/+16 promoter truncation (ΔMSI), and MS II (containing HRE2 and HRE3) was removed by a promoter deletion (ΔMSII) as indicated in Figure 2C. Overexpression of both HIF α isoforms in MCF-7 stable HIF-2 α knock-down (shH2a) cells was used to drive reporter gene expression. HIF α -dependent induction of the ΔMSI construct in MCF-7 shH2a cells was indistinguishable from the -411/+16 construct, indicating that MS I does not contain a functional HRE. In contrast, HIF α -dependent induction of reporter gene activity was strongly reduced when MS II was deleted (Figure 3A).

Deletion of the MS II resulted in basal reporter gene expression comparable to the minimal WISP-2 promoter (-83/+16) activity (Figure 3B). These experiments were repeated in MCF-7 wild-type cells with similar outcome (data not shown). Furthermore, the -83/+16 minimal promoter construct was extended with oligonucleotides containing either wild-type (-112/+16) or mutated (-112/+16mut) HRE2 and HRE3 as indicated in Figure 2C. While no differences in activity were observed between the -112/+16mut and the -83/+16 constructs, a partial but significant reconstitution of the HIF-2α-mediated WISP-2 promoter induction was observed with the -112/+16 construct under hypoxic conditions (Figure 3B). In conclusion, these results demonstrate that HRE2 and/or HRE3 within MS II mainly confer HIF responsiveness to the WISP-2 promoter.

Because MS instability is a hallmark of cancer,[45] we tested whether differences in the length of the CA repeats in MS II might influence HIF α -inducible promoter activity. Therefore, genomic DNA from T-47D cells was amplified and cloned. CA repeat length variants CA_{12}, CA_{13}, CA_{17} and CA_{18} were obtained and compared to the parental -422/+16 construct derived from MCF-7 cells (Figure 3C). However, no change in reporter gene activity could be observed, suggesting that MS instability does not affect HIFα-mediated WISP-2 promoter activity.

Unexpectedly, sequencing of the *WISP2* upstream regulatory region revealed the presence of HRE2 in all breast cancer cell lines analyzed in Figure 1, as well as in Hep3B and HepG2 hepatoma, SK-N-MC neuroepithelioma and HeLa cervical carcinoma, but not in three published sequences (GRCh37.p2, HuRef and Hs_Celera). Only UT-7 megakaryoblastic leukemia cells lacked HRE2 and showed the same sequence like the database entries (data not shown). Therefore, the -252/+16 region was amplified from UT-7 cells (-252/+16_UT7) and compared with the promoter constructs derived from MCF-7 cells. Only a small reduction in response to HIF α overexpression was observed with the -252/+16_UT7 construct (Figure 3D). Also mutation of HRE3 (ΔHRE3) resulted in a modest reduction in reporter gene activity, and a double mutation of HRE2 and HRE3 (ΔHRE2/ΔHRE3) further abrogated reporter gene activity comparable to the one observed after deletion of MS II (Figure 3E). Taken together these results indicate that the two HREs within MS II mediate at least partially HIF α -dependent induction of the WISP-2 promoter.

WISP-2 negatively correlates with tumour infiltration by macrophages

We previously reported that high WISP-2 levels positively correlate with high HIF-2 α levels and prolonged overall survival in breast cancer patients.[8] Because tumour macrophage infiltration, associated with the hypoxic tumour microenvironment and high HIF α levels, not only represents an important step during breast cancer progression but also an important prognostic marker,[46, 47] we analysed whether WISP-2 expression levels are related to tumour-associated macrophage counts. Therefore, over 300 breast cancer samples were immunostained and scored for the

macrophage markers CD68 and CD163 and the pan-leukocyte marker CD45 (Figure 4A). Interestingly, a negative correlation between WISP-2 and tumour-associated macrophage counts was found (Figure 4B). As expected, overall survival of these patients was negatively associated with macrophage infiltration (Figure 4C). These results suggest that HIF-2 mediated WISP-2 expression is a marker for (and maybe is even causally involved in) breast cancer progression stage with low cancer cell proliferation/invasiveness as well as low macrophage infiltration, both contributing to improved prognosis.

HIF modulates the WISP-2 suppressed motility of MCF-7 cells

Silencing of WISP-2 in non-invasive MCF-7 cells has been reported to enhance motility and modulate the expression of genes involved in cancer invasiveness.[30, 48, 49] To investigate the functional consequences of HIF mediated WISP-2 regulation, we stably transfected MCF-7 cells with WISP-2 shRNA, resulting in a robust knock-down of WISP-2 mRNA using two independent shRNA constructs (shW#1 and shW#2) under normoxic as well as hypoxic conditions (Figure 5A). Proliferation of both WISP-2 knock-down cells, but not of previously established HIF-2 α knock-down cells[8] was significantly impaired under both normoxic and hypoxic conditions (Figure 5B). As determined by automated trypan blue exclusion and video microscopy analysis, no difference in gross cell morphology or viability could be observed (data not shown). Low cell density colony formation was somewhat attenuated by knock-down of both HIF-2 α and WISP-2 (Figure 5C). In contrast, anchorage independent colony formation was increased; especially in normoxic cells (Figure 5D). Similarly, recovery from scratches in confluent cell layers was significantly increased by knock-down of both HIF-2 α and WISP-2 (Figure 5E). Probably owing to a remaining weak WISP-2 induction (see Figure 5A), under hypoxic conditions a slightly impaired anchorage-independent colony formation (Figure 5D) as well as scratch recovery of MCF-7 cells was observed (Figure 5E), though the values were still higher than in the hypoxic control cells. In summary, these results are consistent with a role of HIF-2 in the WISP-2 mediated suppression of MCF-7 anchorage independent growth and cell motility, two hallmarks of cancer progression.

5.5 Discussion

In this study, we could corroborate the previously reported hypoxic induction of WISP-2[8] which was HIF-2 dependent in most but not all low invasive breast cancer cell lines tested, suggesting that currently unknown cell type-specific co-factors determine the HIF α isoform responsible for hypoxic WISP-2 induction, rather than intrinsic gene selectivity of the HIF α isoforms themselves. Previous studies suggested a cooperation of HIF-2 α, but not HIF-1 α, with ETS factors resulting in target selectivity[14, 50] but whether a different composition of ETS factors explains the HIF-1 α specific WISP-2 expression in BT-474 cells remains to be explored. Although we observed lower basal levels of WISP-2 promoter activity following mutation of the

conserved CRE, ELK-1 and WRE sites, HIF-2 α mediated WISP-2 regulation remained unaffected, suggesting that none of these factors confers HIF α isoform selectivity.

Two functional HREs were identified which are essential for HIF-2 α specific induction of WISP-2 promoter activity in MCF-7 cells, but even promoter constructs up to 1919 bp upstream of the TSS did not fully recapitulate the hypoxic induction factors determined at the mRNA level. Of note, in a genome-wide chromatin immunoprecipitation study both HIF α isoforms were reported to bind to a region within the first intron of WISP-2[51] suggesting that these elements might contribute to endogenous WISP-2 regulation. However, at least in MCF-7 cells we could not detect any further increase in hypoxic WISP-2 transcription by this region in combination with the upstream regulatory elements (data not shown). The two functional HREs are located within the highly polymorphic MS II region of the WISP-2 promoter. Such unstable polymorphic di-nucleotide repeats are known to play an important role in tumour progression. Microsatellite instability seems to be mostly due to the loss of functional mismatch repair machinery and is implicated in prognosis and therapy response because it alters the expression levels of the affected genes.[45, 52] However, we could not find any role for MS II length in regulating reporter constructs driven by the WISP-2 promoter.

By immunohistochemical analysis of breast cancer samples we recently demonstrated that HIF-2 α and WISP-2 levels correlate with a more differentiated tumour cell type and consistently with a better prognosis.[8] These results are in line with the findings presented herein showing that WISP-2 negatively correlates with tumour macrophage invasion, which provides an additional marker for a better tumour prognosis. Silencing of WISP-2 elevated two parameters of cancer cell aggressiveness: anchorage-independent colony formation as well as recovery from scratches in confluent cell layers. These effects could not simply be explained by changes in proliferation rates and anchorage-dependent colony formation which were actually slightly decreased rather the increased. Interestingly, increased anchorage-independent colony formation and scratch recovery following WISP-2 knock-down could be phenocopied by HIF-2 α silencing. While we cannot exclude that additional signalling pathways are recruited by the hypoxic tumour microenvironment, our data strongly suggest that HIF-2 mediated WISP-2 induction contributes to a less aggressive tumour type.

5.6 Conclusion

Taken together, our data suggest that the previously reported association between high HIF-2 α levels and an increased overall survival rate of breast cancer patients could be explained at least partially by HIF-2 mediated direct induction of WISP-2, maintaining a less aggressive breast cancer phenotype.

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Disclosure

The authors declare no conflicts of interest in this work.

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5.8 Figures

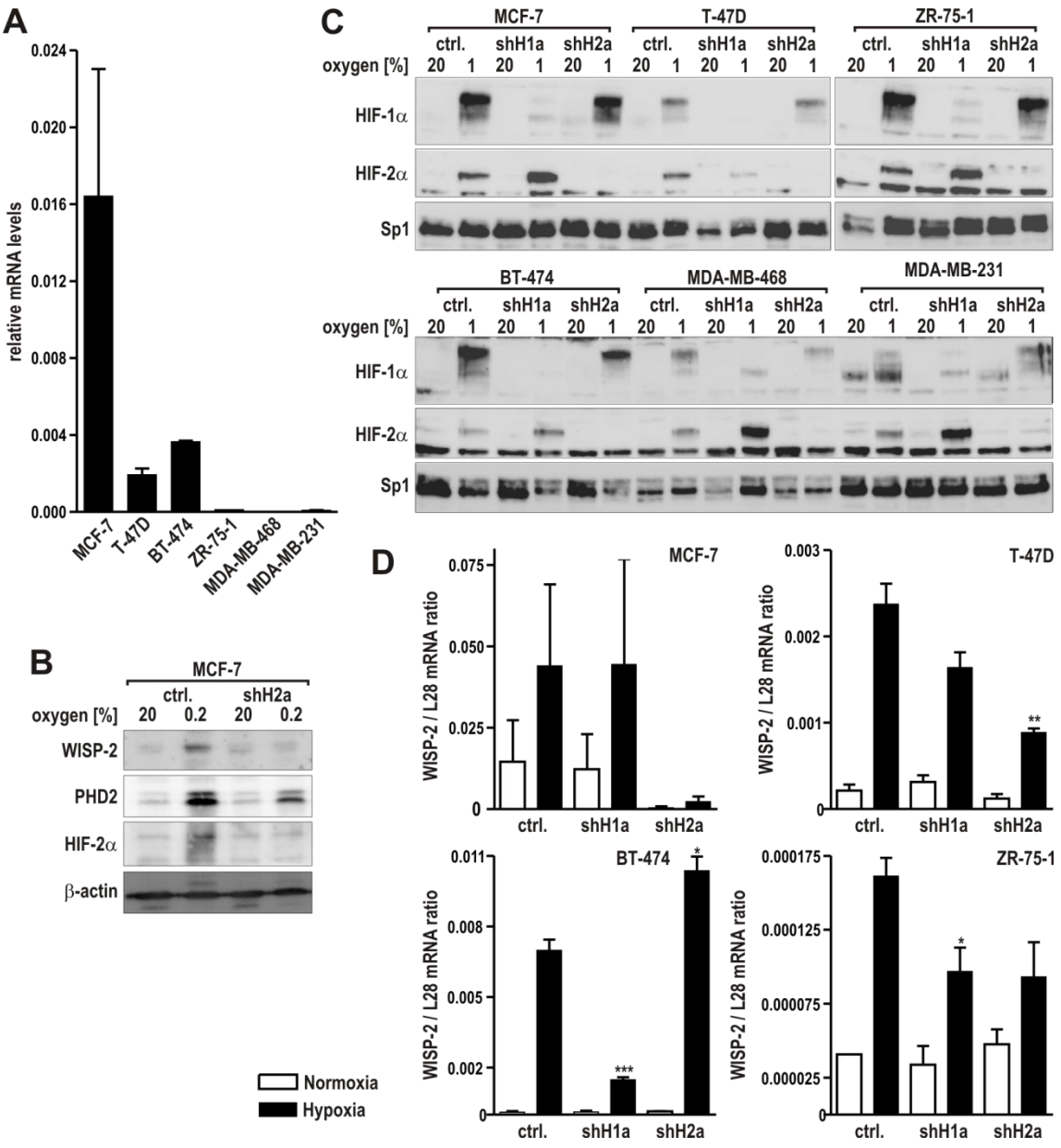

Figure 1 HIF α isoform-specific regulation of WISP-2 transcription. (**A**) Basal WISP-2 mRNA levels were determined by RT-qPCR in the six breast cancer cell lines indicated using a calibrated internal standard. Shown are mean values ± SEM of n = 3 independent experiments. (**B**) MCF-7 cells were cultured in normoxia or exposed to hypoxia for 24 hours, and analysed by immunoblotting using antibodies derived against WISP-2, the HIF target PHD2, HIF-2 α or the constitutively expressed control protein β-actin. (**C**) Cells were stably transfected with either control (ctrl.) shRNA or shRNAs targeting HIF-1 α (shH1a) or HIF-2 α (shH2a) and analysed by immunoblotting of nuclear extracts using antibodies derived against HIF-1 α, HIF-2 α or the constitutively expressed control transcription factor Sp1. (**D**) Hypoxic WISP-2 mRNA induction was determined in the indicated cell lines which were stably transfected with either control (ctrl.) or shRNA constructs as in (**C**). WISP-2 mRNA levels were quantified by RT-qPCR and normalized to the mRNA levels of the ribosomal protein L28. Shown are mean values ± SEM of n = 3 independent experiments. (**D**) For statistical evaluation of the hypoxically exposed cells, the effects of HIF-1 α or HIF-2 α silencing were compared to the control shRNA transfected cells.

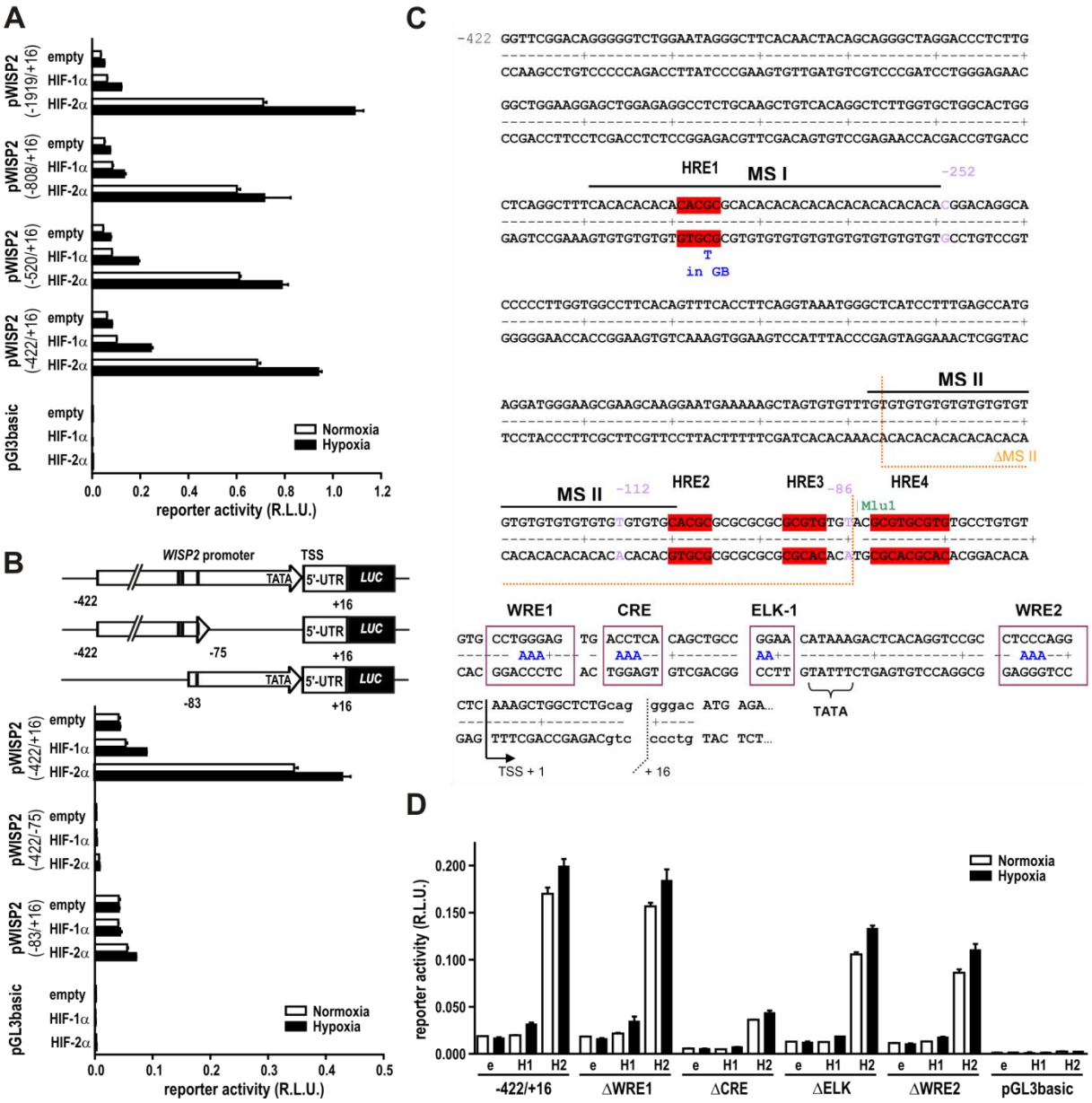

Figure 2 Characterization of the *WISP2* promoter. Dual luciferase reporter gene experiments in MCF-7 cells, transfected with either HIF-1 α or HIF-2 α overexpression vectors together with the indicated firefly luciferase vectors and a renilla luciferase control vector, after 24 hours under normoxic or hypoxic (1% O_2) conditions. (**A**) Promoter truncations from positions -1919 to -422 upstream of the TSS. (**B**) Potential HREs are depicted by black marks (upper panel). HIF responsiveness was lost in the minimal promoter (lower panel). (**C**) Four potential HREs in the *WISP2* 5' flanking region are indicated in red colour. MS, microsatellite repeat; GB, Gene Bank. (**D**) MCF-7 shH2a cells were co-transfected with either the wild-type (-422) promoter construct, or constructs bearing the indicated mutations, together with either an empty (e) vector, a HIF-1 α (H1) or a HIF-2 α (H2) overexpression vector. Shown are mean values ± SD of n = 3 (4 for **D**) independent experiments performed in triplicates. For statistical evaluation, the effects of HIF-1 α and HIF-2 α overexpression have been compared to the respective empty vector controls (**A**, **B**), or were first normalized to the empty vector control values and then compared to the respective normalized values of the wild-type -422/+16 promoter construct (**D**).

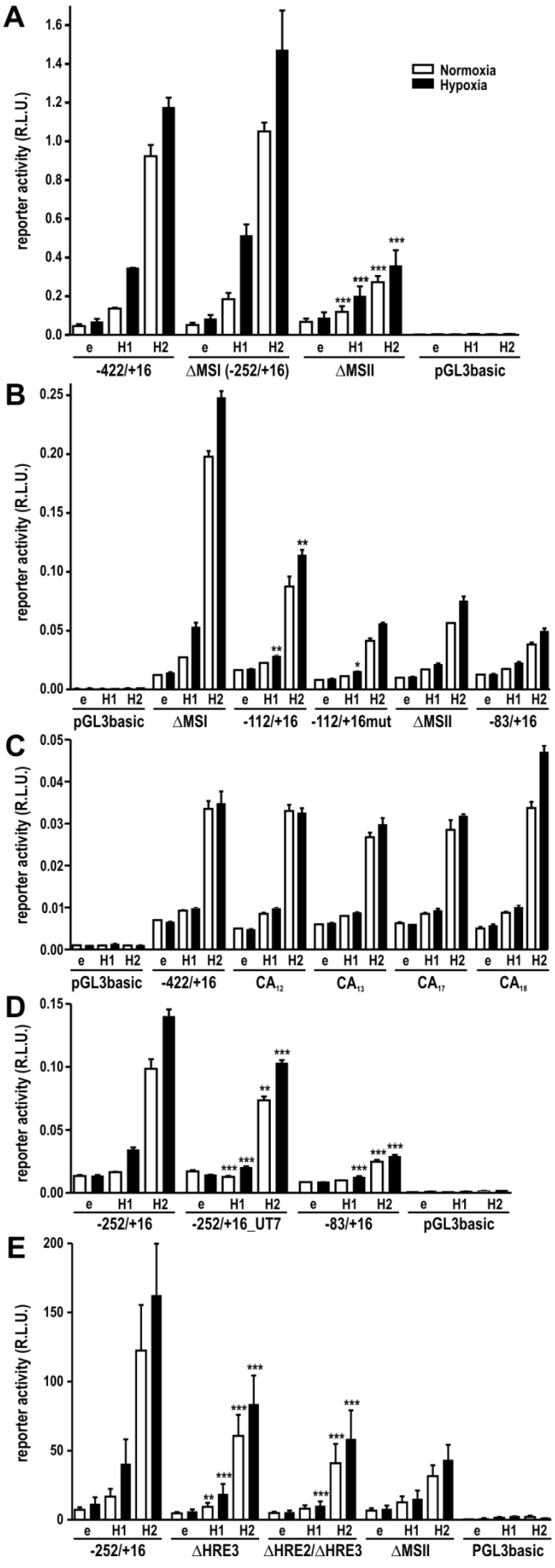
A
Normoxia
Hypoxia
reporter activity (R.L.U.)
e H1 H2
-422/+16
ΔMSI (-252/+16)
ΔMSII
pGL3basic
B
pGL3basic
ΔMSI
-112/+16
-112/+16mut
ΔMSII
-83/+16
C
pGL3basic
-422/+16
CA12
CA13
CA17
CA18
D
-252/+16
-252/+16_UT7
-83/+16
pGL3basic
E
-252/+16
ΔHRE3
ΔHRE2/ΔHRE3
ΔMSII
PGL3basic

Figure 3 Identification of the HREs responsible for HIF-mediated induction of WISP-2 transcription. Dual luciferase reporter gene experiments in MCF-7 shH2a cells, transfected with either an empty (e), a HIF-1 α (H1) or a HIF-2 α (H2) overexpression vector together with the indicated firefly luciferase constructs and a renilla luciferase control vector, after 24 hours under normoxic or hypoxic (1% O_2) conditions. (**A**) WISP-2 promoter constructs lacking either MS I (ΔMSI) or MS II (ΔMSII) were compared with the -422/+16 construct. (**B**) WISP-2 promoter constructs containing wild-type (-112/+16) or mutant HRE2 and HRE3 (-112/+16mut) were compared with the ΔMSI, ΔMSII or minimal promoter (-83/+16) constructs. (**C**) WISP-2 promoter constructs containing the indicated MS repeat length polymorphisms identified in T-47D cells were compared with the -422/+16 fragment derived from MCF-7 cells. (**D**) WISP-2 promoter constructs containing the HRE2-deficient sequence identified in UT-7 cells (-252/+16_UT7) were compared with the corresponding fragment (-252/+16) and the minimal promoter (-83/+16) derived from MCF-7 cells. **A** to **D**) Shown are mean values ± SD of representative experiments performed in quadruplicates. (**E**) WISP-2 promoter constructs containing the -252/+16 fragment, either wild-type (-252/+16), HRE2 mutant (ΔHRE2) or HRE2 and HRE3 double mutant (ΔHRE2/ΔΔHRE3), were compared with the ΔMSII construct. Shown are mean values ± SD of n = 3 independent experiments performed in quadruplicates. For statistical evaluation, the effects of HIF-1 α and HIF-2 α overexpression were first normalized to the empty vector control values and then compared to the respective normalized values of the wild-type -422/+16 (**A**, **C**) or the -252/+16 (**D**, **E**) promoter constructs. (**B**) Similarly, the activities of -112/+16 and -112/+16mut were compared to the -83/+16 minimal promoter.

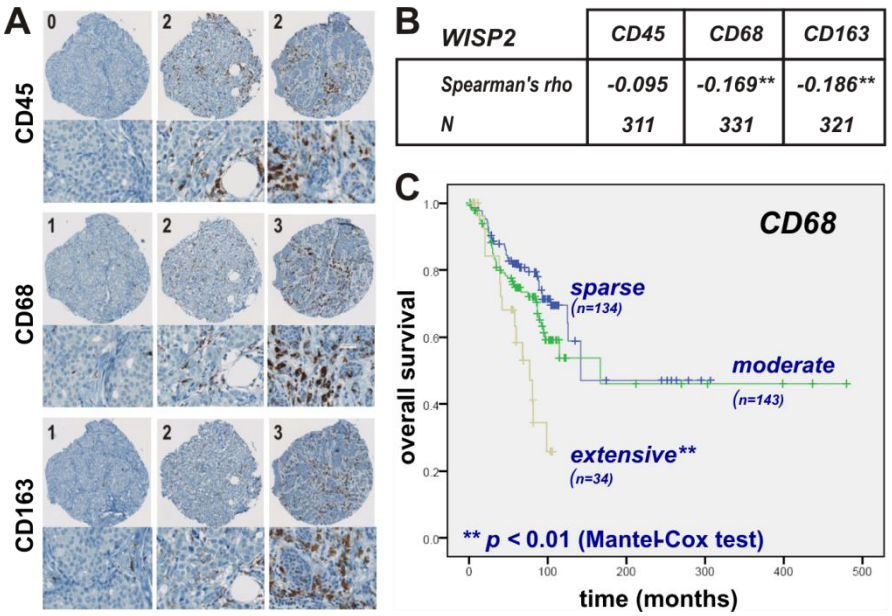

Figure 4 WISP-2 correlations with macrophage infiltration of human breast cancer tissues. (**A**) Exemplary spots of breast cancer tissue micro arrays immunostained for CD45, CD68 and CD163 macrophage markers. Numbers in upper left corners indicate categorization of staining intensity: 0/1, sparse; 2, moderate; 3, extensive. (**B**) Negative correlation of WISP-2 expression with macrophage markers CD68 and CD163 in 331 and 321, respectively, human breast cancer samples. (**C**) Negative prognostic correlation between overall survival of breast cancer patients and high infiltration levels with CD68-positive tumour associated macrophages.

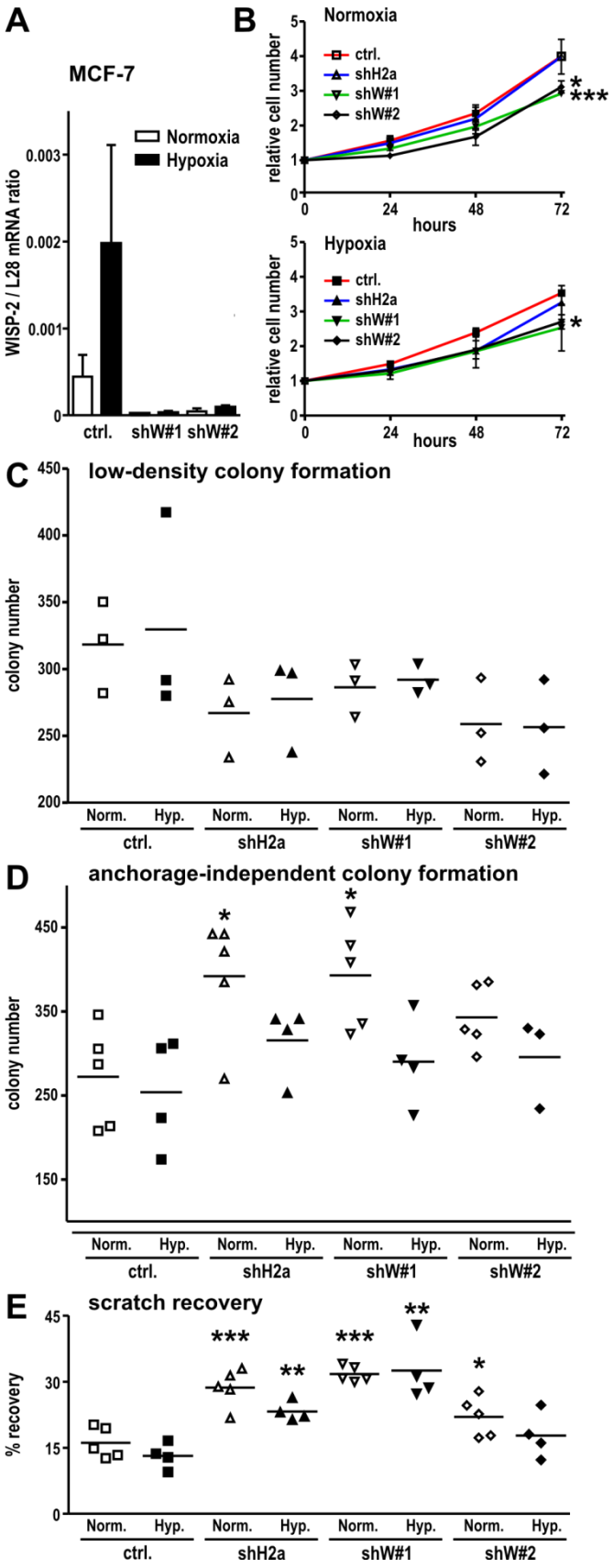
A
MCF-7
Normoxia
Hypoxia
WISP-2 / L28 mRNA ratio
0.003
0.002
0.001
0
ctrl.
shW#1
shW#2
B
Normoxia
ctrl.
shH2a
shW#1
shW#2
relative cell number
hours
*
***
Hypoxia
*
C
low-density colony formation
colony number
450
400
350
300
250
200
Norm.
Hyp.
ctrl.
shH2a
shW#1
shW#2
D
anchorage-independent colony formation
150
E
scratch recovery
% recovery
45
30
15
0
**

Figure 5 Role of HIF-2 α and WISP-2 in MCF-7 proliferation and motility. (**A**) MCF-7 cells were stably transfected by lentiviral infection with control (ctrl.) or two independent WISP-2 shRNA constructs (shW#1 and shW#2). Following normoxic or hypoxic (24 hours, 0.2% O_2) exposure, WISP-2 mRNA levels were determined by RT-qPCR and normalized to the mRNA levels of the ribosomal protein L28. Shown are mean values ± SD of n = 5 independent experiments. (**B**) Proliferation of MCF-7 cells kept under normoxic or hypoxic conditions for up to 3 days was determined by cell counting, and cell numbers were normalized to the number of cells initially seeded. Shown are mean values ± SD of n = 3 independent experiments performed in triplicates. Differences after 3 days relative to the un-transfected controls were analysed by Student's t-test (*p<0.05, ***p<0.001). (**C**) Low density colony formation of MCF-7 cells cultured under normoxic or hypoxic conditions for 10 days. Shown are the colony numbers of n = 3 triplicate measurements and the corresponding mean values. (**D**) Anchorage independent colony formation of MCF-7 cells cultured in soft agar under normoxic or hypoxic conditions for 14 days. Shown are the colony numbers of n = 4 to 5 triplicate measurements and the corresponding mean values. (**E**) Recovery of scratched confluent MCF-7 cell layers after 24 hours of normoxic (Norm.) or 0.2% O_2 hypoxic (Hyp.) exposure. Shown are the results of 4 to 5 independent experiments. (**A** to **E**) For statistical evaluation, the values were compared to the respective control shRNA transfected cells.

6. Unpublished manuscript: WISP-2 and its function in MCF-7

6.1 Introduction

Detection and determination of WISP-2 are complicated due to the nature of WISP-2 as a secreted protein and the previous lack of effective antibodies against WISP-2. Here, I describe several approaches I performed in order to detect and quantify WISP-2 in the MCF-7 breast cancer cell line.

Induction of estrogen-independent growth and promotion of epithelial-to-mesenchymal transition (EMT) have been observed in WISP-2 knocked down breast cancer cells[173]. In addition, microarray gene expression analysis revealed that WISP-2 represses expression of genes associated with EMT[162]. Furthermore, overexpression of WISP-2 in the highly invasive breast cancer cell line MDA-MB-231 results in reduction of proliferation and invasiveness[173]. Together with the biphasic nature of WISP-2 expression in breast cancer cells, these observations suggest the potential roles of WISP-2 as an anti-invasive factor in breast cancer. In this thesis, I aimed to functionally explore the consequences of WISP-2 regulation by hypoxia, therefore I first investigated the mRNA levels of several genes reported to be involved in EMT. The genes I selected are MMP9, E-cadherin, and vimentin.

MMP9 is one of the most intensely investigated matrix metalloproteinase (MMP) and is involved in various physiological and pathophysiological roles. Physiologically, MMP9 has been shown to be essential for embryonic implantation and has also been reported to play a significant role in neovascularisation[238,239]. In pathophysiological conditions involving inflammatory processes, such as rheumatoid arthritis and cancer, MMP9 is overexpressed[240,241]. E-cadherin is a calcium-dependent cell-cell adhesion molecule, a member of the cadherin superfamily and a known tumour suppressor protein[242]. It is well established that the loss of E-cadherin is correlated with tumour progression and that the loss of E-cadherin plays a role in increasing the motility of cancer cells and hence promoting invasion and metastasis[243,244]. In contrast to E-cadherin, which is highly expressed in epithelial cells, vimentin is highly expressed in mesenchymal cells[245]. Vimentin is a type III intermediate filament (IF) protein and is a major cytoskeletal component responsible for the maintenance of cell integrity[245]. Due to its high expression in mesenchymal cells, vimentin, together with E-cadherin, is considered as a marker of cells undergoing EMT[245].

6.2 Materials and methods

6.2.1 Cell culture and reagents

Human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 1% or 10% heat-inactivated foetal calf-serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin (Invitrogen). Hypoxia experiments were performed at the indicated concentration of oxygen (O_2) and 5% carbon dioxide (CO_2) in a gas-controlled glove box (InvivO2 400, Baker Ruskinn, Bridgend, UK).

6.2.2 mRNA detection and quantification

Cellular RNA was extracted using the phenol-chloroform protocol and cDNA was generated by reverse transcription (RT) from 2 µg total RNA using AffinityScript reverse transcriptase (Agilent). Transcript levels were estimated by quantitative (q) PCR using a SybrGreen qPCR reagent kit (Sigma-Aldrich) in a MX3000P light cycler (Agilent). Ribosomal protein L28 mRNA levels were used as normaliser. Primers used are listed in table 1.

Gene	Forward primer	Reverse primer	Amplicon size
MMP9	TTGACAGCGACAAGAAGTGG	GCCATTCACGTCGTCCTTAT	179
E-cadherin	TGCCCAGAAAATGAAAAAGG	GTGTATGTGGCAATGCGTTC	200
Vimentin	GAGAACTTTGCCGTTGAAGC	TCCAGCAGCTTCCTGTAGGT	170
L28	GCAATTCCTTCCGCTACAAC	TGTTCTTGCGGATCATGTGT	198

6.2.3 Immunoblotting

Combined cytoplasmic and nuclear extracts were prepared using the following lysis buffers: TNMG lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 10% glycerol, 1 mM sodium vanadate and protease inhibitor cocktail), NTEN lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2 mM EDTA, 1% Nonidet P-40, 1mM phenylmethylsulfonyl fluoride and 1 x protease inhibitor cocktail), and RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl and 1 x protease inhibitor cocktail). Protein concentration was determined by the bicinchoninic acid assay method and up to 50 µg cellular protein was subjected to immunoblot analysis. Antibodies against the following proteins were used: WISP-2 (Abcam, Santa Cruz, and USCN), HIF-1α (BD Transduction Laboratories, Allschwil, Switzerland), HIF-2α (Novus Biologicals, Littleton, CO, USA), PHD2 and β-actin (Sigma-Aldrich).

6.2.4 Human WISP-2 enzyme-linked immunosorbent assay (ELISA)

Briefly, 1.5 to 2 x 10^6 cells were plated on a 10 cm^2 plate. The day after seeding, medium was replaced with 10 ml DMEM supplemented with 1% FCS without antibiotics and cells were exposed to 20% or 0.2% O_2 for the stated time duration. For the specified experiments, medium was collected and filtered consecutively with filters of three different cut offs (50 kDa, 30 kDa, and 10 kDa) (Amicon Ultra-4 centrifugal filter units). Concentrates and filtrates were collected and tested. Human WISP-2 ELISA (E96894Hu ELISA kit for WISP-2, USCN Ltd) was performed according to the manufacturer's instruction.

6.2.5 Immunofluorescence

1 x 10^5 MCF-7 cells were plated in 12-well plates on top a cover slip. A day later, cells were exposed to hypoxia for 24 hours. Cells were fixed in 0.4% paraformaldehyde in PBS, permeabilised in 0.1% Triton/PBS, blocked with 10% FCS/PBS, and exposed to rabbit-antihuman WISP-2 (A96894Hu, USCN Ltd). Secondary antibody (goat anti-rabbit Alexa488) was used to visualise the cells. DAPI was used to visualise the nuclei. Samples were mounted in Mowiol and visualised using fluorescence microscope.

6.2.6 Motility and Boyden chamber assays

Briefly for motility assay, cells were grown to full confluency in 12-well plates. Following crosswire scratching with a 200 µl pipette tip, the cells were exposed to normoxia or hypoxia in FCS-free DMEM for 8 hours. For the treatment with recombinant WISP-2, 10 nM of recombinant WISP-2 was added to the cells right after scratching and exposed to normoxia or hypoxia. The cell-free area was measured and converted to percent recovery. To determine cell migration, a chamber (Corning Incorporated) containing an 8 µm pore size polycarbonate filter insert that divides the chamber into upper and lower portions was used. Briefly, 1 x 10^5 cells were seeded to the upper chamber of the insert, while the lower chamber was filled with 10% FCS supplemented DMEM. After allowing the assays to proceed for 20 hours under normoxia or hypoxia, the cells on the top of the filter were removed by swabbing and the remaining cells on the bottom of the filter were stained with Giemsa and counted under a light microscope.

6.2.7 Statistical analysis

If not indicated otherwise, unpaired Student's t-tests were applied. Differences between two values were considered statistical significant if $p < 0.05$. Asterisks shown in the figures indicate the following levels of significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

6.3 Results

6.3.1 Detection of WISP-2 on MCF-7

As WISP-2 is known to be a secreted protein, I performed ELISA to detect secreted WISP-2 **(Figure 6.1)**. I confirmed the specificity of the ELISA by applying 1 ng recombinant WISP-2 protein in DMEM (1% FCS) and compared with control DMEM (1% FCS). I also attempted to determine the best lysis buffer for WISP-2 protein extraction and I observed higher WISP-2 levels using RIPA lysis buffer compared to TNMG and NTEN lysis buffers.

To detect secreted WISP-2, I collected the supernatants from MCF-7 cells incubated for 24, 48, 72, 96, and 120 hours under normoxia. At the same time, I also collected supernatants from MCF-7 cells incubated under normoxia or hypoxia for 72 hours. I filtered the collected supernatants consecutively with filters of three different cut offs and applied both the concentrates and the ultrafiltrates on ELISA. I observed an increase in signals. However, I cannot conclude that the detected signals are derived from WISP-2 due to the inability of the filtration processes to differentiate the signals, suggesting the possibility of nonspecific binding of the antibody against non-WISP-2 protein. The reported size of WISP-2 is 29 kDa and I expected to observe higher WISP-2 levels at the concentrate after filtration with 10 kDa filter. Lastly, I performed ELISA on protein extracts from manipulated breast cancer cell lines. I did observe lower levels of the supposedly WISP-2 on shHIF-2α MCF-7 and MDA-MB-231; however the shWISP-2 MCF-7 (#62) did not show reduced WISP-2 levels.

I also performed immunofluorescence experiments to detect WISP-2 in MCF-7 and MDA-MB-231. However, I could not differentiate the signal since we detected signals also in the supposedly WISP-2 knocked down (KD) and negative cell lines, such as shWISP-2 and MDA-MB-231 **(Figure 6.2)**.

6.3.2 The role of WISP-2 in the aggressiveness of breast cancer cells

As mentioned previously, WISP-2 has been shown to be particularly relevant in human breast cancer due to its biphasic expression nature. Functionally, the silencing of the WISP-2 gene results in reduced serum-dependent breast tumour cell proliferation[173]. Furthermore, the overexpression of this protein in highly invasive MDA-MB-231 results in reduced cell proliferation and invasiveness[173]. Based on these observations, we hypothesised a possible role of O2-regulated EMT by WISP-2. We first investigated the changes of three EMT markers: MMP-9, E-cadherin and vimentin by HIF-1α and HIF-2α **(Figure 6.3a)**. I then investigated the regulation of these EMT markers by WISP-2 **(Figure 6.3b)**. Furthermore, I treated MCF-7 with recombinant WISP-2 at different time-course and different concentrations. Unfortunately, I could not observe any changes in the levels of EMT markers upon treatment with recombinant WISP-2.

To study the functional roles of WISP-2, scratch recovery and Boyden chamber assays were performed **(Figure 6.4)**. Consistent with the previously published

recovery of confluent cells after 24 hours, scratch recovery in confluent cell layers was significantly increased by knockdown of both HIF-2α and WISP-2 after 8 hours. To further confirm the roles of WISP-2 in cell motility, scratch recovery assays were performed with the addition of 10 nM recombinant WISP-2. Treatment with 10 nM recombinant WISP-2 did not result in significant changes in the scratch recovery rate. Moreover, Boyden chamber assay was utilised to investigate the roles of WISP-2 in the migratory capabilities of MCF-7. I observed a significant increase in migrated cells with the knockdown of HIF-2α. In general, I also observed an increase in cell migration for the knockdown of WISP-2 (both #62 and #64); however, the increases were not significant.

6.4 Discussions

As a secreted protein, WISP-2 protein detection presents a challenge, especially with the previous lack of specific antibodies. To complement immunoblotting I utilised multiple techniques such as ELISA and immunofluorescence in order to detect WISP-2. ELISA is an ideal method in detecting secreted protein as supernatant from MCF-7 cells can be collected and tested directly without further manipulation. I encountered difficulties with the usage of 10% FCS in the DMEM used with the maintenance of MCF-7. The 10% FCS is required for the optimum growth of MCF-7 cells; however it complicated the ELISA protocol with the introduction of multiple non-human proteins and resulted in background signals during WISP-2 detection. At the same time, MCF-7 cells need the presence of 10% FCS as the absence of FCS resulted in increased cell deaths. As such, I decided to perform the experiments in the presence of 1% FCS. With this, I reduced the presence of non-human proteins and simultaneously maintain MCF-7 cells in a better condition.

Knock down of WISP-2 in breast cancer cells has been reported to induce estrogen-independent growth and promotion of EMT. In this thesis, I explored the potential role of WISP-2 in EMT. Functional assays such as scratch recovery and Boyden chamber assays were performed. Knock down of HIF-2α and WISP-2 in MCF-7 cells resulted in increased motility and migration, two characteristics of EMT. However, investigation of the levels of MMP9, E-cadherin and vimentin on HIF-2α-KD and WISP-2 KD MCF-7 cells did not indicate the role of these genes in the increased motility and migration of MCF-7 cells. Hence my observations contradict the previously reported role of WISP-2 in EMT.

To further confirm the reported role of WISP-2, I treated MCF-7 cells with recombinant WISP-2. The rationale is that if knockdown of WISP-2 in breast cancer cells promoted EMT, then the presence of WISP-2 might reverse this and hence promoted mesenchymal-to-epithelial transition (MET). Treatment of MCF-7 cells with recombinant WISP-2 at different concentrations or different time-points did not result in changes in MMP9, E-cadherin and vimentin, again contradicting the role of WISP-2 in EMT. Moreover, treatment of MCF-7 with recombinant WISP-2 did not affect the

motility of MCF-7 cells. One could argue that the recombinant WISP-2 did not assert its role due to the fact that it might not be internalised into the cells. To counter this argument, overexpression of WISP-2 must be performed, followed by the quantification of gene changes and functional assays.

6.5 References

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6.6 Figures

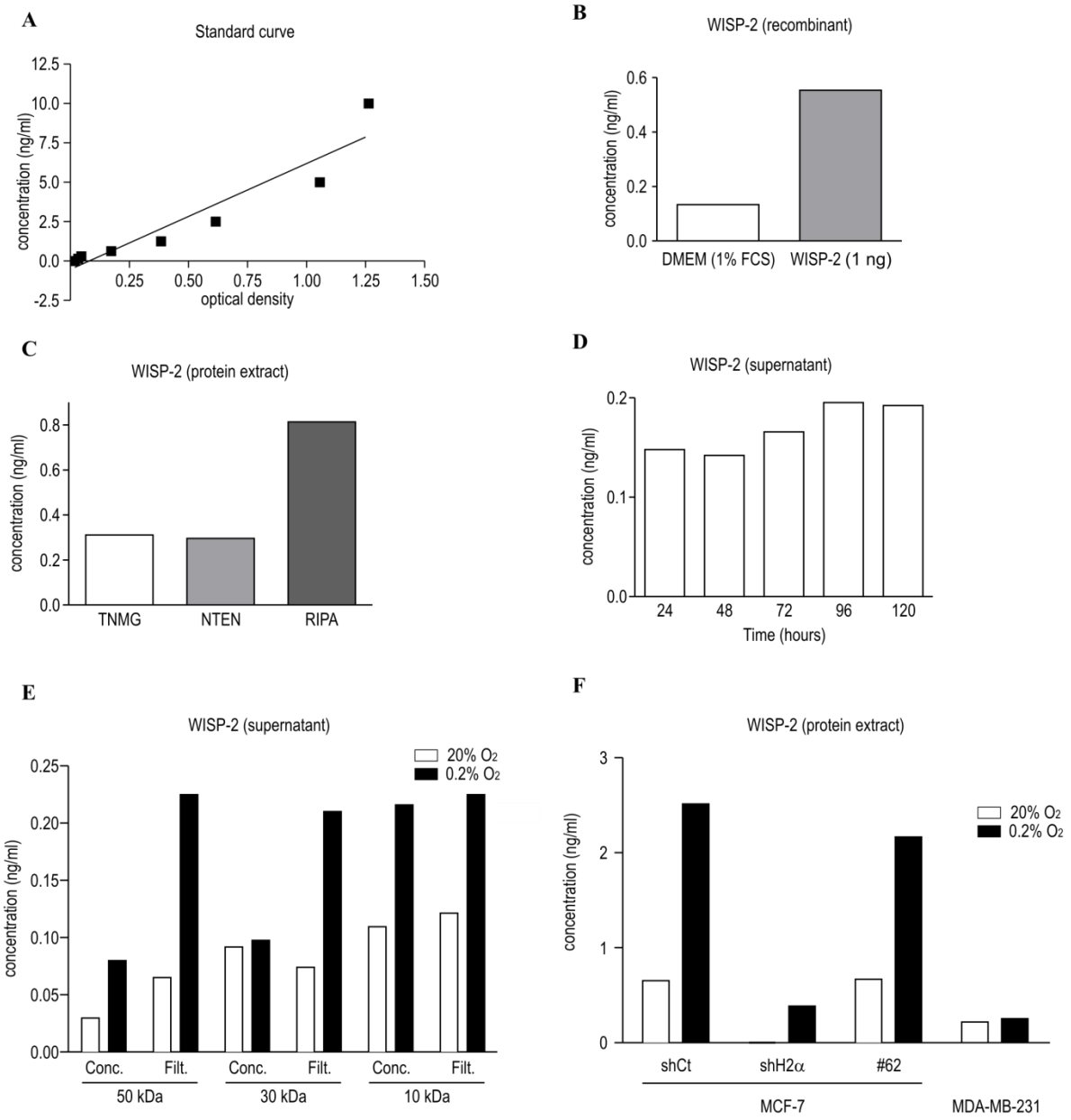

Figure 6.1 ELISA was performed to detect WISP-2 (A) Standard curve of ELISA. **(B)** The specificity of the ELISA was tested by applying 1 ng recombinant WISP-2 **(C)** Different extraction protocols were used to determine the best lysis buffers for the extraction of WISP-2 protein from MCF-7 **(D)** Time-dependent accumulation of WISP-2 in the supernatant of MCF-7 cells **(E)** Supernatant of MCF-7 cells incubated for 72 hours and filtered were tested for WISP-2 protein by ELISA. Conc. = concentrate of filtration protocol, Filt. = filtrate from the filtration protocol **(F)** ELISA was performed on protein extracts from MCF-7 shControl (shCt), shHIF-2α (shH2α), shWISP-2 (#62) and MDA-MB-231.

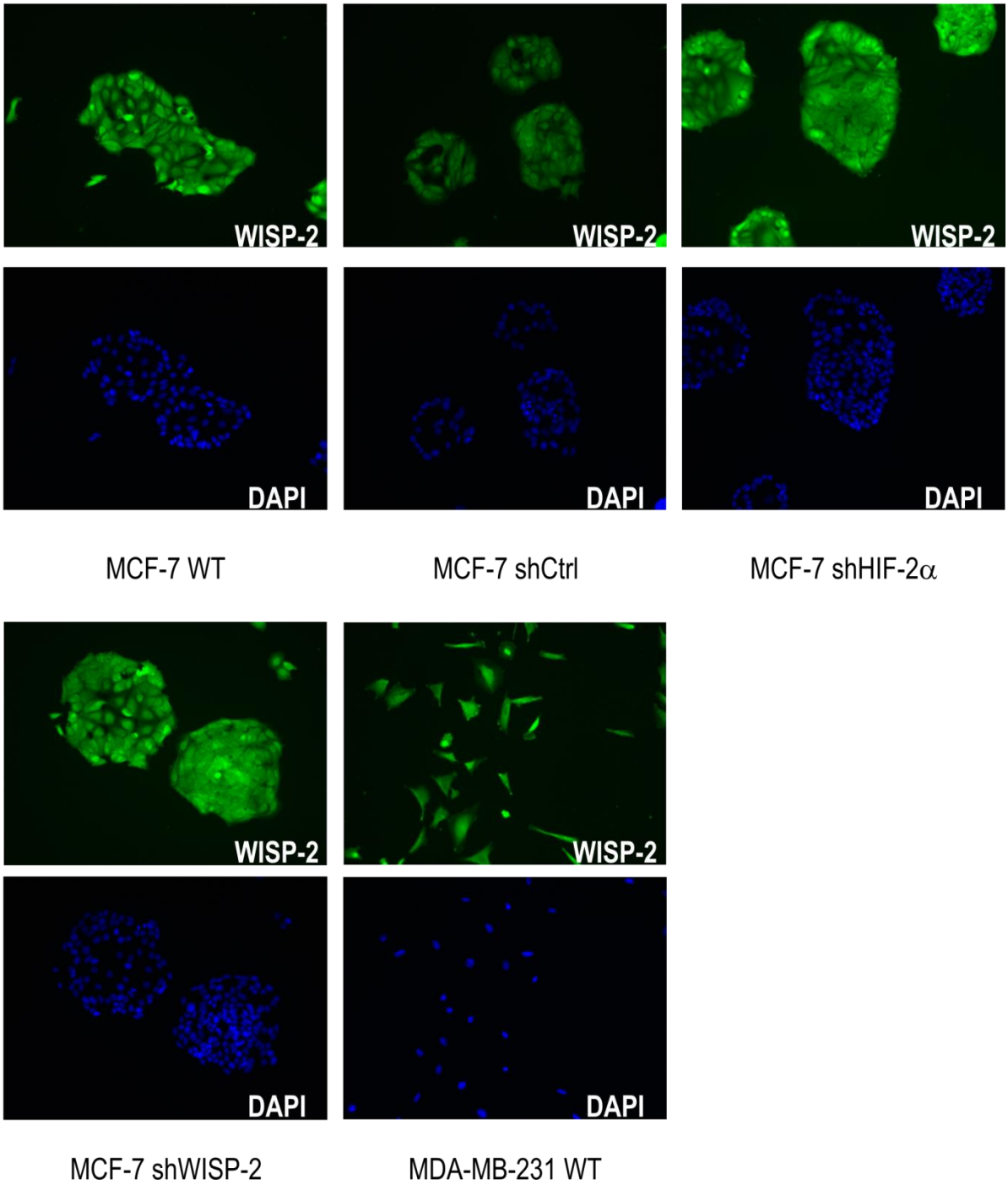

Figure 6.2 Immunofluorescence detection of WISP-2 in MCF-7 cells. MCF-7 WT, shControl (shCtrl), shHIF-2α, shWISP-2 and MDA-MB-231 WT cells were exposed to hypoxia (0.2% O_2) for 24 hours. Cells were then fixed and treated with rabbit-antihuman WISP-2. DAPI was used to stain the nuclei.

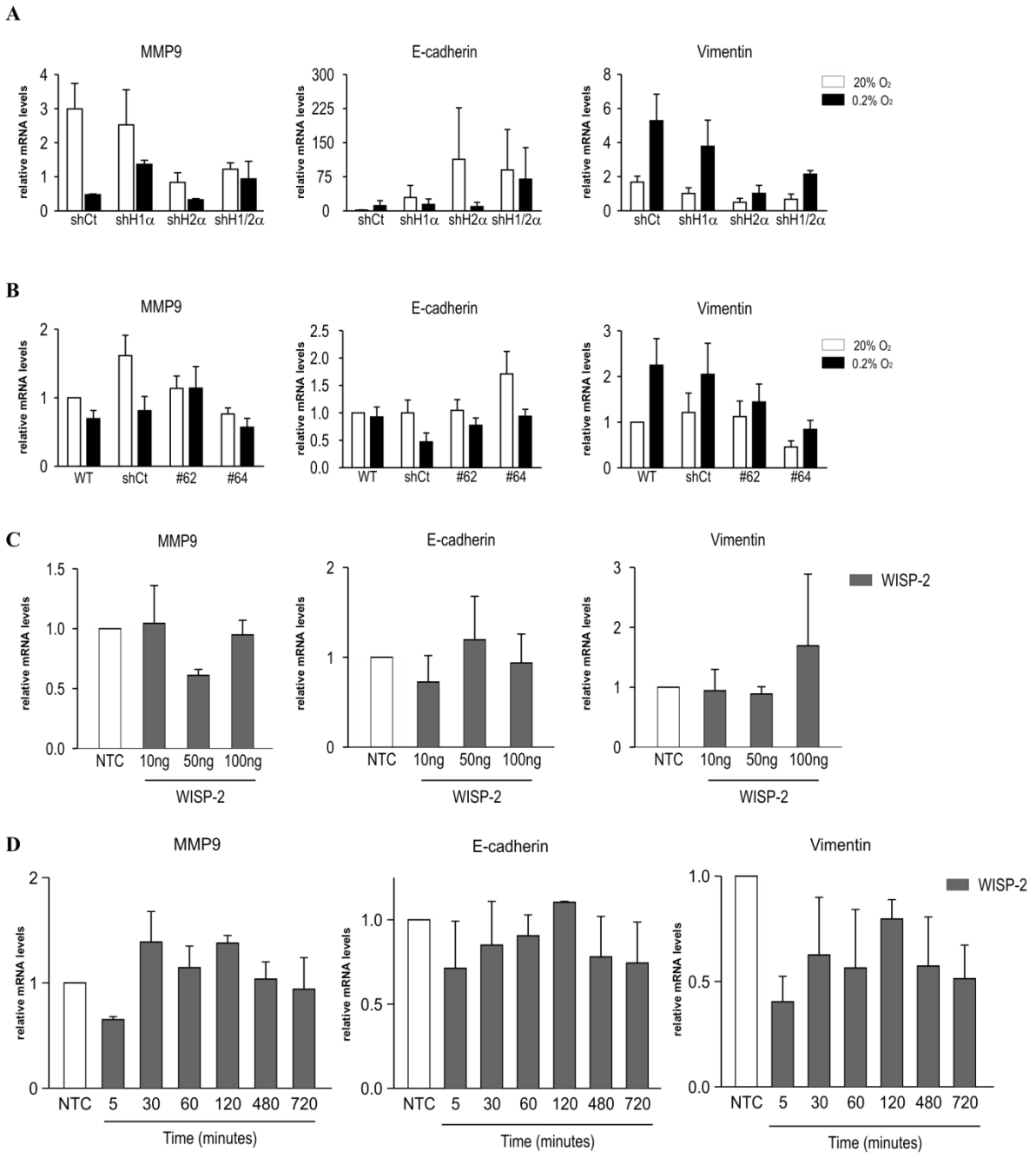

Figure 6.3 The effects of WISP-2 on epithelial-to-mesenchymal transition (EMT). MMP9, E-cadherin, and vimentin were selected as markers for EMT. The mRNA levels of MMP9, E-cadherin and vimentin were quantified in **(A)** MCF-7 shCtrl (shCt), shHIF-1α (shH1α), shHIF-2α (shH2α), shHIF-1/2α (shH1/2α), and **(B)** two different MCF-7 shWISP-2 (#62 and #64) under normoxia or hypoxia. **(C)** MCF-7 cells were treated with different concentrations of recombinant WISP-2 for 24 hours. **(D)** 10ng recombinant WISP-2 was applied on MCF-7 cells at different time points. Shown are mean values ± SEM of n=3 independent experiments.

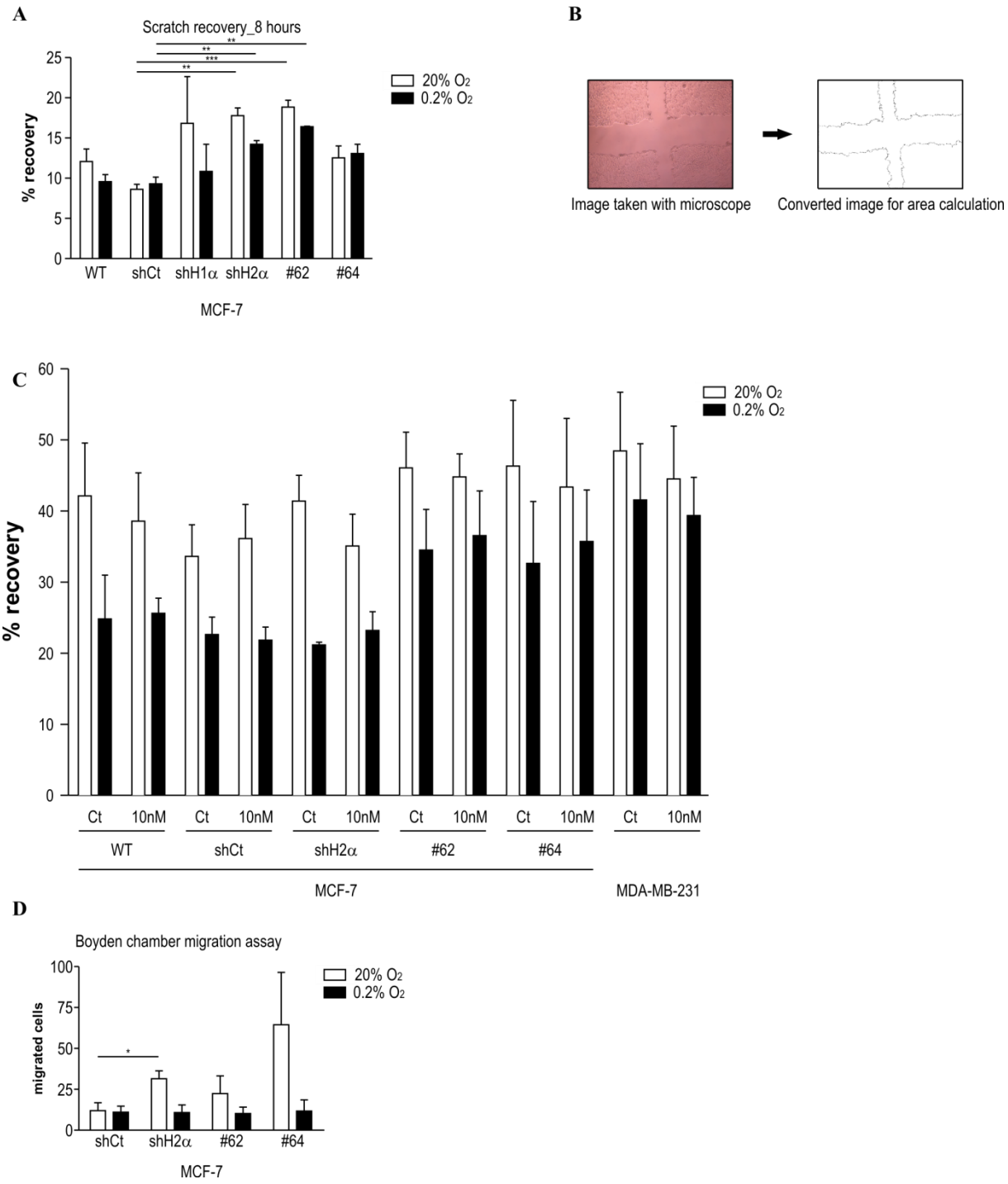

Figure 6.4 Modulation of motility and migration by WISP-2. (A) Confluent cells were scratched and incubated for 8 hours under normoxia and hypoxia. **(B)** Scratched areas were measured before and after incubation and percentage recovery was calculated. **(C)** Cells were also treated with 10 nM recombinant WISP-2 for 24 hours. **(C)** Cell migration was investigated using Boyden chamber migration assay. Shown are mean values ± SEM of n=3 independent experiments.

7. Manuscript II: Estrogen-dependent downregulation of hypoxia-inducible factor (HIF)-2α in invasive breast cancer cells

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Keywords: ERα, HER2, histone deacetylation, hormone therapy, tumor oxygenation

7.1 Abstract

The involvement of estrogen (E2) and hypoxia in the tumor progression is well established. Hypoxia has been reported to activate and degrade estrogen receptor alpha (ERα) in breast cancer cells. Furthermore, E2 has been shown to regulate hypoxia-inducible factor (HIF)-1α protein, but its role in HIF-2α regulation remains largely unexplored. In this study, we found that both HIF-2α mRNA and protein were down-regulated in ER-positive but not ER-negative breast cancer cells upon treatment with E2. The analysis of 690 samples derived from 608 mixed and 82 triple-negative breast cancer patients revealed that high HIF-2α tumor levels are associated with a better prognosis in human epidermal growth factor receptor 2 (HER2)-positive patients. Consistently, hormone receptor and HER2-positive breast cancer cells displayed less pronounced downregulation of HIF-2α by E2. Experiments using histone deacetylase (HDAC) inhibitors indicate that the E2 mediated decrease in HIF-2α mRNA is due to transcriptional repression. A functional estrogen response element (ERE) was identified in the first intron of *EPAS1*, suggesting transcriptional co-repressor recruitment by ERα. Our results demonstrate a novel modulation of HIF-2α in breast cancer cells and might partially explain the association between high HIF-2α and better prognosis in triple-positive breast cancer patients.

7.2 Introduction

The ability of cancer cells to adapt to micro-environmental tissue hypoxia is mainly mediated by hypoxia-inducible factors (HIFs), which affect every aspect of cancer progression, comprising metabolism, proliferation, inflammation, angiogenesis, metastasis and therapy resistance[1-4]. Transcriptionally active HIFs are heterodimers composed of a constitutively expressed β subunit and an oxygen labile HIF-1α or HIF-2α subunit, the stability and activity of which is regulated by prolyl-4-hydroxylase domain (PHD) and factor inhibiting HIF (FIH) enzymes[5]. Despite showing similar protein structures and having identical DNA recognition sequences, distinct - sometimes even opposite - functional roles of HIF-1α and HIF-2α in tumorigenesis have been reported[6-9]. We and others found different kinetics of hypoxic HIF-1α and HIF-2α protein induction and target gene expression[10,11], further suggesting non-overlapping roles for these two related transcription factors.

Estrogens are steroid hormones and represent the primary female sex hormones, regulating diverse physiological processes in reproductive, mammary, cardiovascular, osseous, hepatic, and neuronal tissues[12-16]. The cellular effects of estrogen are mediated by two estrogen receptor (ER) isoforms, ERα and ERβ, which belong to the family of nuclear hormone receptors[17]. Ligand binding leads to the dissociation of heat shock proteins from the ER, which is followed by receptor dimerization and nuclear translocation. In the nucleus, the activated dimer complex binds to estrogen response elements (EREs) located within the regulatory regions of target genes[18].

Besides regulating numerous aspects of human physiology, estrogens also influence diverse pathophysiological processes, including the onset and progression of breast cancer[19]. Breast cancer is the most common cancer in women worldwide and the second most common cancer overall. Approx. 1.7 million new cases were diagnosed in 2012, which represents 12% and 25% of all new cancer cases and all cancers in women, respectively[20,21]. The presence of elevated ERα levels in benign breast epithelium correlates with an increased risk of breast cancer, suggesting a role for ERα in breast cancer initiation[22]. 17β-estradiol (E2), the dominant circulating estrogen, regulates the growth of many breast tumors and approx. 70% of breast cancers express ERα. Most of these ERα-positive tumors depend on estrogen signaling for their growth and survival[23].

In conjunction with estrogen, hypoxia has been reported to play an important role in the development and progression of breast cancer[24-28]. In breast cancer cells, estrogen and hypoxia modulate the expression of genes involved in proliferation, differentiation, angiogenesis, metabolism and apoptosis[29-31]. Further studies revealed the presence of a cross-talk between estrogen signaling pathways and HIF-1α regulation in breast cancer[28,32]. Estrogen has been reported to rapidly induce ERα-c-Src-PI3K interactions which activates the PI3K/AKT/mTOR pathway and subsequent HIF-1α protein translation by phosphorylation of the p70 S6 kinase and 4EB-P1[27]. Activation of G protein-coupled receptor GPR30 (GPER) by E2 triggered the GPER/EGFR/ERK/c-fos signaling pathway, leading to increased VEGF via HIF-1α upregulation[33]. Furthermore, ERα has been reported to directly induce HIF-1α transcription, which might modulate the anti-estrogen response in breast cancer treatment[32]. In contrast, ERβ has been reported to play an opposing role to ERα. Transcriptional activity of HIF-1 is inhibited by ERβ, which is mediated by ubiquitin-dependent degradation of HIF-β[27].

Despite the numerous studies on HIF-1α regulation by estrogen, the interaction between estrogen signaling and HIF-2α regulation is currently unknown. By immunohistochemical detection of HIF-2α in tissue microarrays of 282 invasive breast cancer cases we previously found that patients expressing high HIF-2α levels had a better overall survival rate compared to patients expressing low HIF-2α[11]. Our study was confirmed by the designation of HIF-2α in a list of genes associated with favorable outcome based on studies with different cohorts of breast cancer patients[34].

Here, we report a previously not recognized regulation of HIF-2α by estrogen, suggesting an inverse interplay between estrogen and HIF-2α signaling, which might be involved in breast cancer progression.

7.3 Results

E2 downregulates HIF-2α mRNA and protein levels in ERα-positive but not ERα-negative breast cancer cell lines

We previously reported a HIF-2 specific regulation of WISP-2 expression in breast cancer cells[35]. Because WISP-2 is a known ERα target gene[36], we aimed for the analysis of the cooperation between estrogen and oxygen signaling. Therefore, the time-dependent effect of E2 treatment on HIF signaling in MCF-7 cells was investigated. Unexpectedly, HIF-2α mRNA levels were progressively reduced with increasing time of E2 treatment (Figure 1A). Maximal inhibitory effects were reached after 12 to 24 hours, and the latter time-point was selected for all subsequent experiments, also based on our previous observations that HIF-2α protein levels in MCF-7 cells are expressed maximally after 24 hours of hypoxic exposure[11].

We further tested the effects of E2 on HIF-2α in additional breast cancer cell lines, including another luminal-like ERα-positive (T-47D), a basal B-like ERα-negative (MDA-MB-231), and a basal A-like ERα-negative (MDA-MB-468) cell line. Both, HIF-2α mRNA (Figure 1B) and hypoxically stabilized protein (Figure 1C) levels were downregulated in the ERα-positive MCF-7 and T-47D but not in the ERα-negative MDA-MB-231 and MDA-MB-468 cell lines. Exposure to hypoxia (0.2% O_2) for 24 hours generally did not affect the HIF-2α mRNA levels, except in MDA-MB-231 cells where HIF-2α mRNA was induced. However, E2 did not alter this cell type-specific HIF-2α mRNA regulation. Neither HIF-1α mRNA (Figure 1B) nor hypoxically stabilized HIF-1α protein levels (Figure 1C) were significantly affected by 24 hours E2 treatment. These data suggest that HIF-2α is specifically downregulated by E2-ERα signaling on the mRNA level which resulted in corresponding changes on the protein levels.

The selective estrogen receptor modulator tamoxifen prevents ERα-dependent HIF-2α downregulation

To further investigate the involvement of estrogen signaling in HIF-2α and HIF-1α regulation, MCF-7 cells were treated for 24 hours with the ERα-specific agonist propyl pyrazole triol (PPT) and a selective agonist for the GPR30 E2 receptor (G-1) under normoxic or hypoxic conditions. HIF-2α but not HIF-1α mRNA levels were downregulated by PPT but not by G-1 (Figure 2A). Progesterone receptor (PgR) was

included as a positive control for ERα activation by PPT. These results were confirmed in T-47Dcells (Figure 2B).Similar data were obtained on the protein level (Figure 2C), suggesting the involvement of ERα but not GPR30 in estrogen-mediated HIF-2α inhibition.

The current first line therapy of ER-positive breast cancer patients is the treatment with the selective estrogen receptor modulator (SERM) tamoxifen. Therefore, MCF-7 cells were treated with E2, with or without tamoxifen, under normoxic or hypoxic conditions. As shown in Figure 2D, tamoxifen at least partially prevented HIF-2α mRNA downregulation by E2, while HIF-1α mRNA levels were neither affected by E2 nor tamoxifen. *Vice versa*, PgR mRNA upregulation by E2 was partially prevented by tamoxifen. As in Figure 1A, hypoxia inhibited PgR induction, probably due to the known degradation of ER protein by hypoxia in MCF-7 cells as shown below[37]. Corroborating the findings on the mRNA levels, tamoxifen also partially prevented the E2-mediated downregulation of the hypoxically stabilized HIF-2α protein levels (Figure 2E). The slight HIF-2α but not HIF-1α protein downregulation by tamoxifen may be explained by the similarly decreased mRNA levels. However, these findings suggest that SERMs can reverse the inhibitory effects of estrogen on HIF-2α.

Inverse correlation between HIF-2α and ERα mRNA levels in breast cancer

To complement the pharmacological E2 receptor modulation shown above, ERα was downregulated by siRNA treatment of MCF-7 cells. Knockdown efficiency was confirmed by a 96% decrease of the constitutive ERα mRNA levels as well as by a 97% decrease of the E2-induced mRNA levels of the ERα target gene PgR (Figure 3A). Both, basal and E2-inhibited HIF-2α mRNA levels were significantly increased in siERα but not siCtrl treated MCF-7 cells. While E2 strongly downregulated HIF-2α mRNA levels in siCtrl cells, the remaining slight downregulation in siERα cells was not significant (Figure 3A). Similar results were obtained in MCF-7 cells stably transfected with three independent shRNA constructs which resulted in 74-83% ERα knockdown efficiency and in a strong decrease of PgR, whereas constitutive HIF-2α mRNA levels were enhanced (Figure 3B). Probably due to the remaining ERα, blockade of the E2-mediated HIF-2α downregulation and PgR upregulation was rather inefficient in shERα compared with siERα cells (data not shown). These findings were corroborated on the protein level where hypoxically stabilized HIF-2α

but not HIF-1α is clearly less downregulated by E2 in siERα than in siCtrl treated MCF-7 cells (Figure 3C).

Because the results shown above suggest an inverse correlation between HIF-2α and ERα mRNA levels in breast cancer cell lines, we explored transcriptome data from various clinical breast cancer studies employing the R2 microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi). A study of 66 tamoxifen-treated breast cancer patients showed a significant negative correlation between the expression of HIF-2α and ERα (Figure 3D). Several additional datasets from three different microarray studies including 136, 116 and 61 patients, respectively, breast cancer patients displayed a similar inverse correlation (supplementary Figure 1).

HIF-2α is a positive prognostic factor in HER2-positive breast cancer

To study the role of HIF-2α in breast tumorigenesis, two tissue microarrays containing invasive breast cancer tumor samples derived from 690 breast cancer patients with primary breast cancer were immunostained for HIF-2α. The tissue microarrays contained areas from invasive breast cancer belonging to ERα-positive, HER2-positive or triple-negative cases, as described previously[38]. The signals from the invasive breast cancer samples in the tissue microarrays were scored based on the presence or absence of cytoplasmic and nuclear HIF-2α staining as exemplified in Figure 4A. When all 690 cancer cases were included, neither cytoplasmic nor nuclear HIF-2α correlated with overall survival (Figure 4B). Stratification of cytoplasmic and nuclear HIF-2α to nodal status, tumor stage and histological grade showed no correlation with overall survival (supplementary Figures 2A-F). Also the analysis of the 82 triple-negative breast tumor samples did not reveal any significant association between HIF-2α levels and overall survival (supplementary Figure 2G). However, stratification of nuclear HIF-2α according to the HER2 positivity status in hormone receptor-positive breast cancer patients displayed a significant direct correlation with overall survival (Figure 4C).

Because the ERα-positive breast cancer cell lines used in Figure 1 are not overexpressing HER2, we analyzed the hormone receptor and HER2-positive breast cancer cell line BT-474. While treatment with 10 nM E2 for 24 hours downregulated HIF-2α mRNA levels (Figure 4D), the fold inhibition was less pronounced than in

MCF-7 and T-47D cells (Figure 1), and cannot be detected on the protein level (Figure 4E). Diminished downregulation of HIF-2α by E2 in HER2-high ERα-positive BT-474 cells is consistent with the correlation between high HIF-2α levels and better survival of hormone receptor and HER2-positive breast cancer patients.

ERα-dependent HIF-2α downregulation is independent of mutual HIFα inhibition

To address the functional mechanism underlying HIF-2α inhibition by E2/ERα, we investigated known candidate HIF-2α inhibitors. We and others previously reported the mutual inhibition of HIF-1α and HIF-2α[7,39]. Because E2 has been shown to induce HIF-1α expression[33], we hypothesized that increased HIF-1α might subsequently decrease HIF-2α. This hypothesis was tested by adding E2 to stably HIF-1α or HIF-2α depleted MCF-7 cells. As expected, shHIF-1α MCF-7 cells express higher levels of HIF-2α mRNA, whereas shHIF-2α had no effect on HIF-1α mRNA levels in this cell line (Figure 5A). Both, PgR and ERα remained largely unaffected by either HIF-1α or HIF-2α knockdown, and E2 downregulated HIF-2α in shHIF-1α to the same extent as in shCtrl cells. Also CITED-2, a preferential HIF-2 target gene[10,40], was still downregulated by E2 in shHIF-1α MCF-7 cells (Figure 5A). Similar results were obtained on the protein level (Figure 5B), suggesting that mutual HIFα inhibition does not play any role in E2/ERα-dependent HIF-2α downregulation.

Histone deacetylation is involved in HIF-2α inhibition by E2

Histone deacetylation is one of the prerequisites for chromatin remodeling and regulation of gene expression. In order to study the role of histone deacetylation in HIF-2α transcriptional regulation, MCF-7 cells were treated with E2 in the absence or presence of the class I and II mammalian histone deacetylase (HDAC) inhibitor trichostatin A (TSA). While TSA alone had no effect, it prevented HIF-2α mRNA (Figure 6A) and protein (Figure 6B) downregulation by E2, suggesting that HDACs are involved in HIF-2α regulation by E2.

According to published chromatin immunoprecipitation-sequencing (ChIP-seq) data[17], ERα binds to four distinct regions within the first intron of the gene encoding HIF-2α (*EPAS1)* upon E2 treatment (supplementary Figure 3A). *In silico* analysis revealed that these regions contained conserved estrogen response element (ERE)

binding motifs. All four regions displayed robust DNaseI hypersensitivity (reflecting open chromatin) and the methylated and acetylated histone marks H3K4Me1 and H3K27Ac, respectively (reflecting active enhancers), but not H3K4Me3 (reflecting active promoters) in MCF-7 cells (supplementary Figure 3B). Based on ChIP-seq information deposited in the UCSC-integrated ENCODE database, ERE4 also displayed binding of ERα in T-47D breast cancer cells. Interestingly, transcription factor (TF) ChIP-seq data further revealed the binding of GATA-2 and GATA-3, established transcriptional repressors[41-44], at ERE3 and ERE4 (supplementary Figure 3B). Moreover, ERα also binds to an ERE within the *HIF1A* gene, overlapping with GATA-3 binding (data not shown).

To analyze the involvement of ERE 1 to 4 in E2 regulation of *EPAS1* gene expression, we evaluated their potential to regulate SV40 promoter-driven firefly luciferase reporter gene expression. The reporter gene constructs were transiently transfected into MDA-MB-231 cells together with an ERα overexpression vector. Transfected cells were treated with E2 for 24 hours under normoxic or hypoxic conditions and the luciferase activities determined. While ERE1 and ERE2 had no effects, ERE3 and ERE4 significantly enhanced E2-induced reporter gene activity (Figure 6C).

Plasmids containing ERE3 and ERE4 were then transfected into MCF-7 cells with or without ERα, GATA-2 or GATA-3. Whereas co-transfection of the reporter genes together with GATA overexpression vector alone did not result in the activation of luciferase activities upon E2 treatment, co-overexpression of ERα resulted in significant E2-dependent activation of luciferase activity (Figure 6D). Taken together, these results indicate that E2-activated ERα locates to at least one ERE within the *EPAS1* gene and recruits several transcriptional co-factors, including GATA factors and HDACs, leading to transcriptional repression of the *EPAS1* gene.

7.4 Discussion

Cross-talk between estrogen signaling and hypoxia-dependent signaling pathways has previously been reported, focussing on the interactions between estrogen signaling and HIF-1α regulation[27,32,37,45,46]. In the present study, we report for the first time the association between estrogen signaling and HIF-2α regulation. Estrogen signaling is an essential component of breast cancer progression as indicated by the prevalence of ERα overexpression in breast cancer patients[47]. Hypoxia represents another major factor in breast cancer progression, and the interaction between these two signaling pathways hence is of major clinical importance[4].

In this study, we observed an ERα-dependent downregulation of HIF-2α mRNA levels by E2. Cell lines with different ERα status, pharmacological and RNA interference experiments confirmed the requirement of ERα for the E2 effects on HIF-2α. Higher constitutive expression of HIF-2α both on the mRNA and protein levels in ERα depleted MCF-7 was phenocopied in microarray data of breast cancer patients with different ERα levels. This observation suggests a constitutive ERα-dependent suppression of HIF-2α expression, which is strengthened by hormonal ERα activation. Of note, the E2-induced HIF-2α repression was almost completely abrogated in hormone receptor and HER2 triple-positive cells. While it is currently unclear how HER2 interferes with HIF-2α regulation, HER2 signalling is known to induce HIF-1α by PI3K/Akt/mTOR signalling[48-50], and a similar mechanism might also overcome E2-mediated HIF-2α repression.

The ERα utilizes multiple mechanisms to either induce or suppress transcription of its target genes, which include direct binding of ligand-activated receptor to the DNA at the EREs, followed by recruitment of transcriptional co-regulators[51,52]. Also an indirect modulation via sequestration of general transcriptional components has been suggested[53]. ERα activation is usually assumed to be associated with increased gene expression, however in fact almost 70% of the genes regulated by E2 are down-regulated in MCF-7[54]. Our observation of HIF-2α down-regulation by E2 is thus consistent with the majority of genes being downregulated upon E2 treatment of MCF-7 cells.

Currently, we have no definitive explanation for the mechanism by which ERα inhibits HIF-2α. The state of histone acetylation is a good predictor of gene activity, and HDACs are known to repress gene expression by modulating the conformational

state of the chromatin. Furthermore, HDACs have been reported to be involved in tumorigenesis[55,56]. Estrogen-mediated repression of the cell cycle inhibitor Reprimo (RPRM) required the interactions between ERα, HDAC7 and FoxA1[57]. In addition, expression of the gene encoding human uridine diphosphate glucuronosyltransferase (*UGT1A*) was inhibited in the presence of ERα. ChIP assays further demonstrated the recruitment of ERα, HDAC1 and HDAC2 to the xenobiotic response elements of *UGT1A* promoters during gene repression[58]. Moreover, tamoxifen-bound ERα has been reported to recruit HDAC to silence gene transcription of ERα targets[59]. Interestingly, tamoxifen treatment of MCF-7 cells has recently been shown to significantly increase HDAC1 binding on the ERE of HIF-1α[32]. In our study, treatment of MCF-7 cells with E2 and TSA abolished ERα-dependent HIF-2α regulation. We identified several EREs within the regulatory region of the *EPAS1* gene, and ERE4 interacts with ERα in both MCF-7 and T-47D cells. ERE4 can also interact with GATA-2 and GATA-3, consistent with recent studies revealing substantial enrichment of GATA3 binding to ERα occupied DNA regions[60,61]. Furthermore, besides inducing gene expression, GATAs have been reported to also exert repressive functions[41-44]. Luciferase reporter gene assays demonstrated a functional interaction between GATA-2/3 and the *EPAS1* ERE4, suggesting a role of ERE4 in ERα-dependent HIF-2α regulation.

In conclusion, hormone activation may lead to ERα dimerization, binding to *EPAS1* ERE4, recruitment of transcriptional co-factors, including GATAs and HDACs, and repression of gene expression. The lack of ERα in triple-negative breast cancer allows for constitutively higher HIF-2α and prevents estrogen-mediated HIF-2α downregulation seen in ERα positive breast cancer. Specifically in triple-positive HER2 overexpressing breast cancer, the downregulation of HIF-2α upon E2 treatment was less pronounced on the mRNA level and was absent on the protein level, which might partially explain the correlation with prolonged overall survival.

7.5 Material and Methods

Reagents

E2 (17β-estradiol), PPT (propyl pyrazole triol), tamoxifen and fulvestrant were purchased from Sigma-Aldrich (St Louis, MO, USA). G-1 was purchased from Tocris Biosciene (Bristol, UK). Reagents were dissolved in ethanol or DMSO. Antibodies against the following proteins were used: HIF-1α (BD Transduction Laboratories, Allschwil, Switzerland), HIF-2α (immunoblotting: Abnova Corporation, Taiwan; immunohistochemistry: Abcam, Cambridge, UK), ERα (Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (Sigma-Aldrich).

Cell culture and treatments

The human breast cancer cell lines MCF-7, T-47D, MDA-MB-231, MDA-MB-468 and BT-474 were cultured in high-glucose Dulbecco's Modified Eagle's Medium (Sigma-Aldrich). Before experiments, the cells were maintained in phenol red-free DMEM supplemented with 10% charcoal-treated FCS (Gibco, Thermo Fischer Scientific, Waltham, MA, USA) for 1 to 2 days. Cells were treated with E2 or vehicle control (0.1% ethanol or 0.1% DMSO) alone or in combination with other ligands for 24 hours under normoxic and hypoxic conditions. Hypoxic experiments were performed at 0.2% oxygen and 5% CO_2 in a gas-controlled glove box (Invivo2 400, Baker Ruskinn, Bridgend, UK) as described previously[62].

mRNA and protein detection

Total cellular RNA was extracted as previously described. Total RNA (2 μg) was reverse transcribed (RT) using AffinityScript reverse transcriptase (Agilent, Santa Clara, CA, USA) and complementary DNA (cDNA) levels were estimated by quantitative polymerase chain reaction (qPCR) using the primers listed in supplementary Table 1 and a SYBR® Green qPCR reagent kit (Sigma-Aldrich) in a MX3000P light cycler (Agilent). Transcript levels were calculated by comparison with a calibrated standard and expressed as ratios relative to ribosomal protein L28 mRNA levels. Immunoblotting and breast cancer tissue microarray analysis were performed as previously described[35,38].

Plasmid construction and reporter gene assays

DNA fragments containing the *EPAS1*-derived EREs were generated by PCR using the primers listed in supplementary Table S1, and cloned into the pGL3-promoter luciferase reporter vector (Promega Corporation, Madison, WI, USA). A cDNA encoding human ERα cloned into the pCMV5 mammalian expression vector was

kindly provided by A. Odermatt (Basel, Switzerland). Human GATA-2, GATA-3, and GATA-4 cloned into pcDNA3.1 were kindly provided by C. Dame (Berlin, Germany). Dual luciferase reporter gene assays were performed as described previously[63].

Statistical analysis

If not indicated otherwise, unpaired Student's *t-tests* were applied. Differences between two values at the $P<0.05$ level were considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

The authors declare no conflicts of interest in this work.

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Supplementary Information

Supplementary Information accompanies the paper.

7.6 References

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7.7 Figures

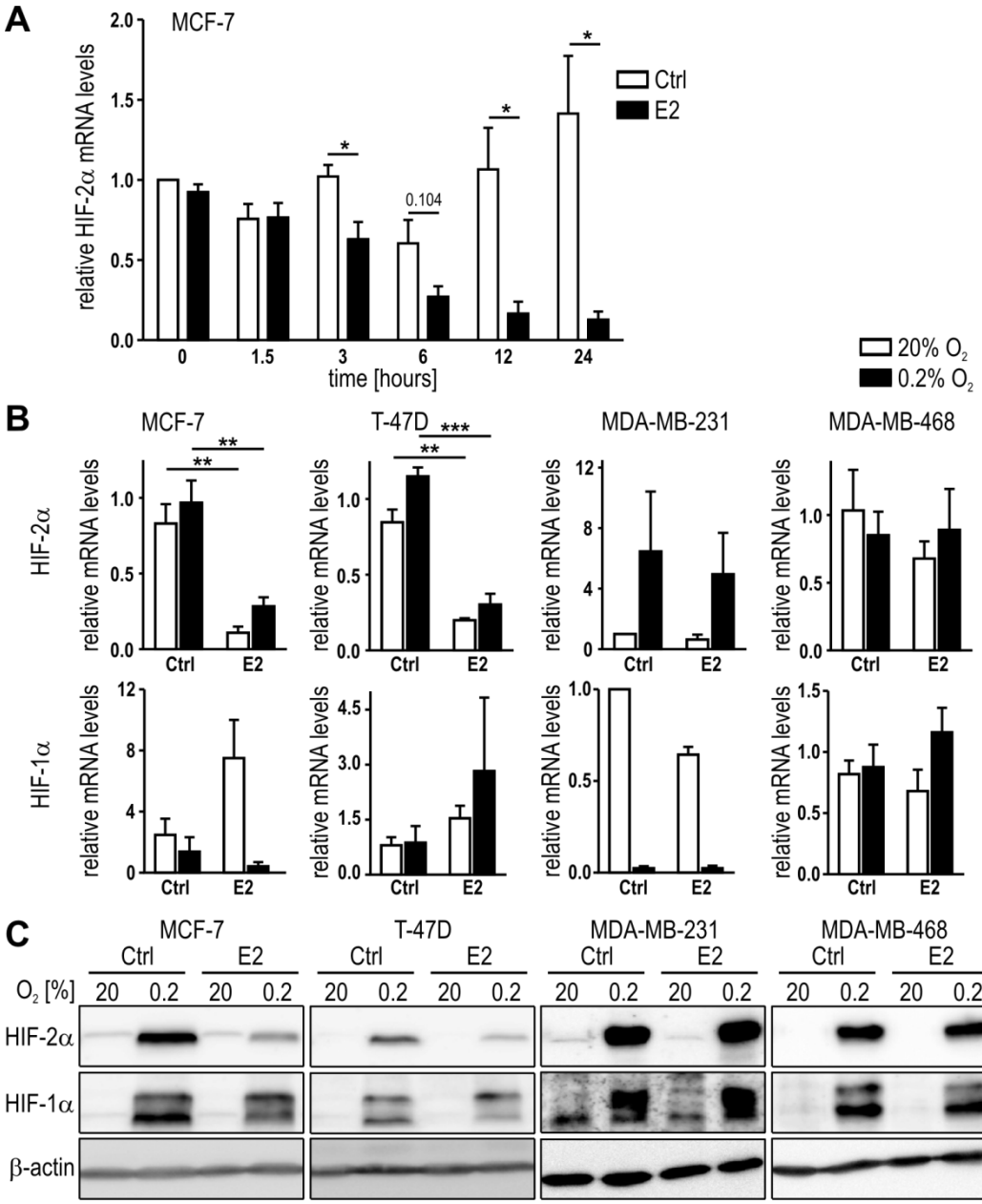

Figure 1: HIF-2α regulation by estrogen in breast cancer cell lines. A. MCF-7 cells were treated with 10 nM E2 for the indicated time periods. HIF-2α mRNA levels were quantified by RT-qPCR, corrected for the endogenous L28 mRNA levels and normalized to the starting time point. **B.** ERα-positive (MCF-7 and T-47D) and ERα-negative (MDA-MB-231 and MDA-MB-468) breast cancer cell lines were treated with 10 nM E2 for 24 hours under normoxic or hypoxic conditions. HIF-2α mRNA levels were quantified as above. Shown are mean values ± standard errors of the mean (SEM) of three to four independent experiments. For statistical evaluation, the effects of E2 treatment were compared with the control (Ctrl) treatment. **P*<0.05; ***P*<0.01; ****P*<0.001. **C.** HIF-2α protein was detected by immunoblotting and constitutively expressed β-actin was used as loading and blotting control.

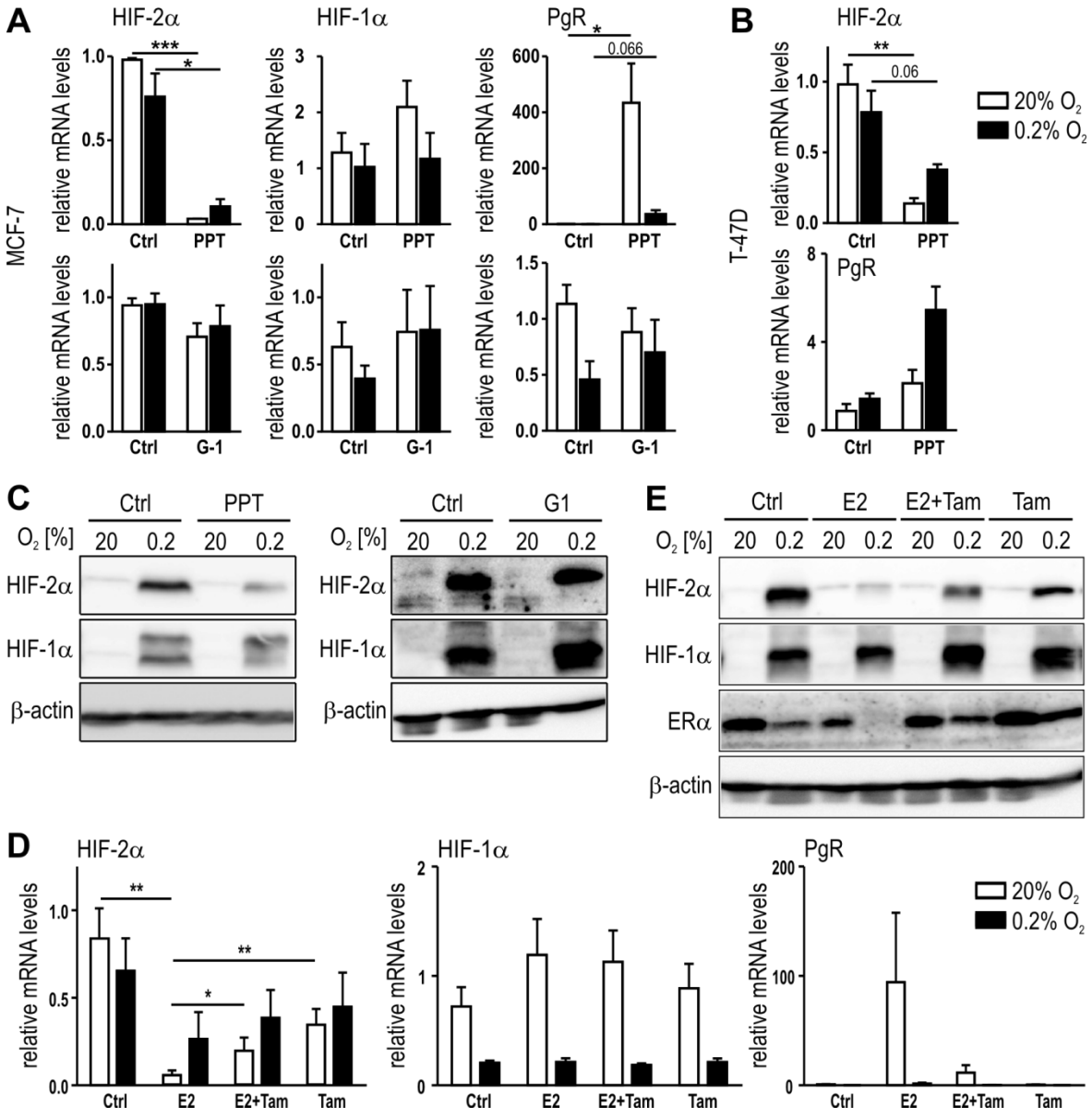

Figure 2: ERα-mediated regulation of HIF-2α by estrogen. MCF-7 (**A** and **C**) or T-47D (**B**) cells were treated for 24 hours with 10 nM PPT, an ERα-specific agonist with approx. 400 times higher affinity towards ERα than towards ERβ, or 1 μM G1 (MCF-7 only), a GPER-specific agonist, under normoxic or hypoxic conditions. The mRNA (**A** and **B**) and protein (**C**) levels were determined by RT-qPCR and immunoblotting, respectively. **E.** and **D.** Tamoxifen (5 μM) was added to MCF-7 cells with or without 10 nM E2 and mRNA (**D**) and protein (**E**) levels were determined. Shown are mean mRNA values ± SEM of three independent experiments. For statistical evaluation, the effects of E2 treatment were compared with control (Ctrl) treatment. **P*<0.05; ****P*<0.001.

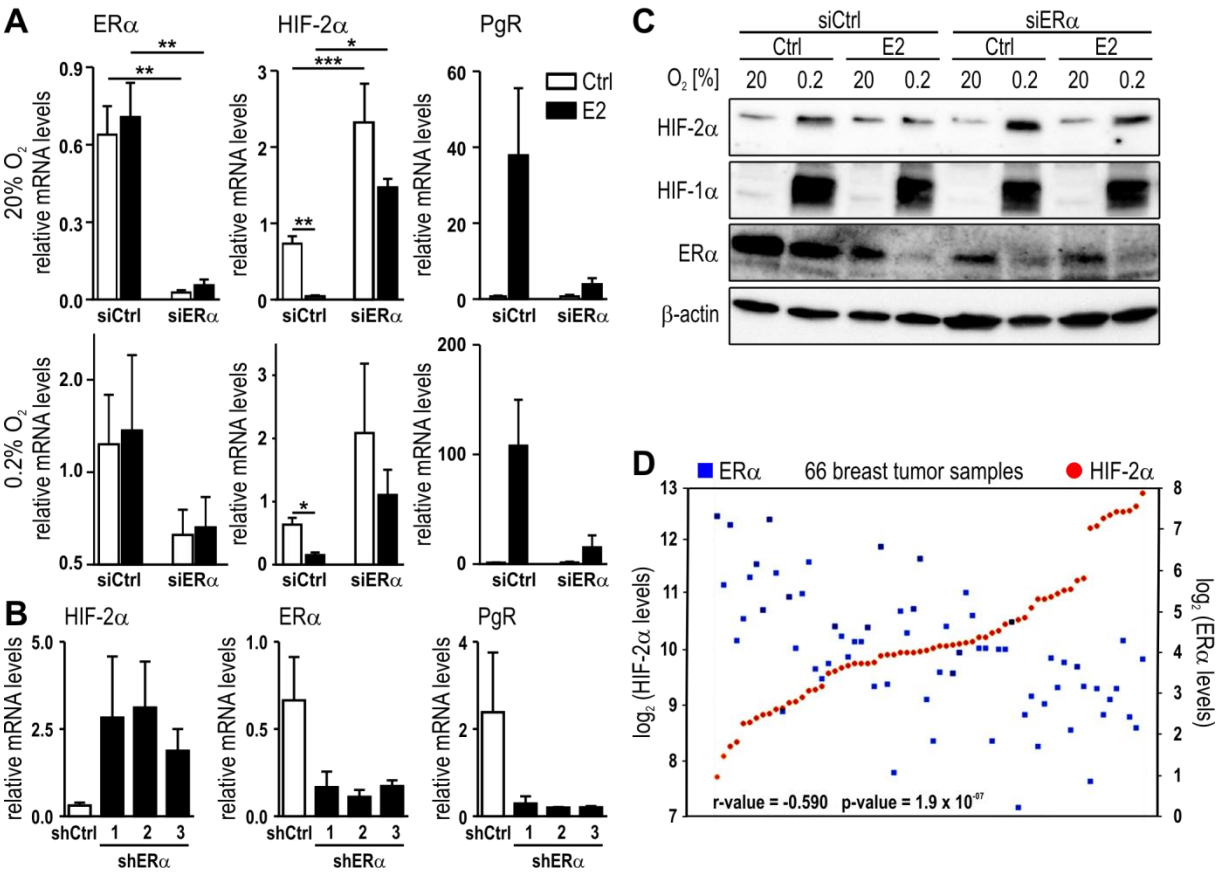

Figure 3: Reciprocal regulation of HIF-2α and ERα. ERα was knocked down in MCF-7 cells using siERα (**A** and **C**) or shERα (**B**) and then treated with 10 nM E2 under normoxic or hypoxic conditions. mRNA (**A** and **B**) and protein (**C**) levels were subsequently determined by RT-qPCR and immunoblotting, respectively. Shown are mean mRNA values ± SEM of three independent experiments. For statistical evaluation, the effects of ERα silencing were compared with siCtrl cells; the effects of E2 treatment were compared with control (Ctrl) treatment. **P*<0.05; ***P*<0.01. **D.** Microarray data from public databases were compiled using the R2 genomic analysis tool. Significance of the negative correlation between HIF-2α (red dots) and ERα (blue squares) mRNA levels was assessed by one-way ANOVA.

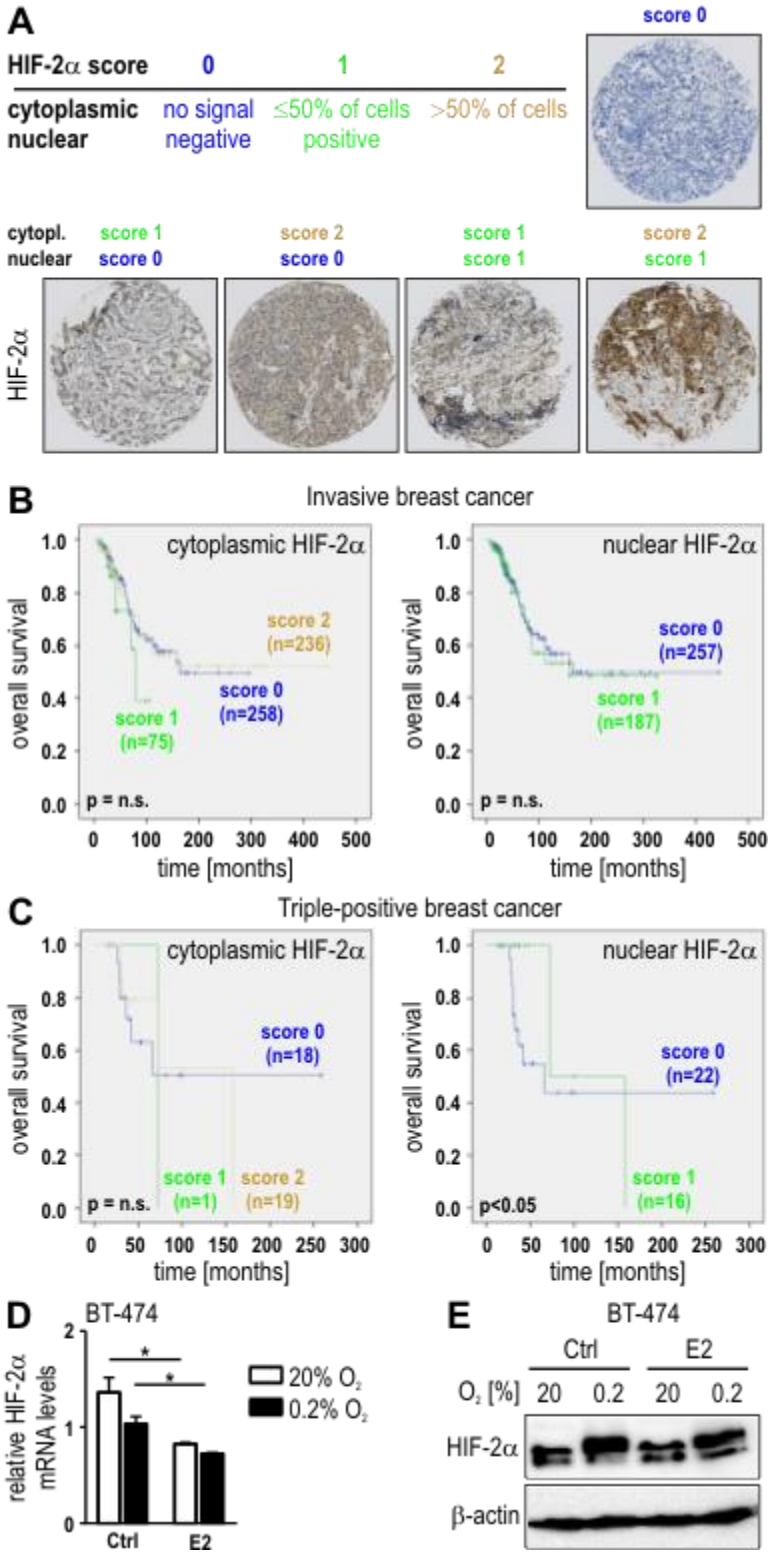

Figure 4: HIF-2α as a prognostic factor in HER2-positive breast cancer. A. Scoring system and representative images for cytoplasmic and nuclear HIF-2α staining of invasive breast cancer tissues. **B.** HIF-2α was scored in tissue microarrays of 608 invasive breast cancer cases (excluding triple-negative cases) and displayed in Kaplan-Meier survival curves. **C.** Survival curves of hormone receptor-positive and HER2 overexpressing breast cancer patients according to HIF-2α scores. **D.** and

E. Triple-negative BT-474 breast cancer cells were treated with 10 nM E2 for 24 hours under normoxic or hypoxic conditions, and mRNA (**D**) and protein (**E**) levels of HIF-2α were determined. Shown are mean mRNA values ± SEM of three independent experiments. For statistical evaluation, the effects of E2 treatment were compared with control (Ctrl) treatment. **P*<0.05.

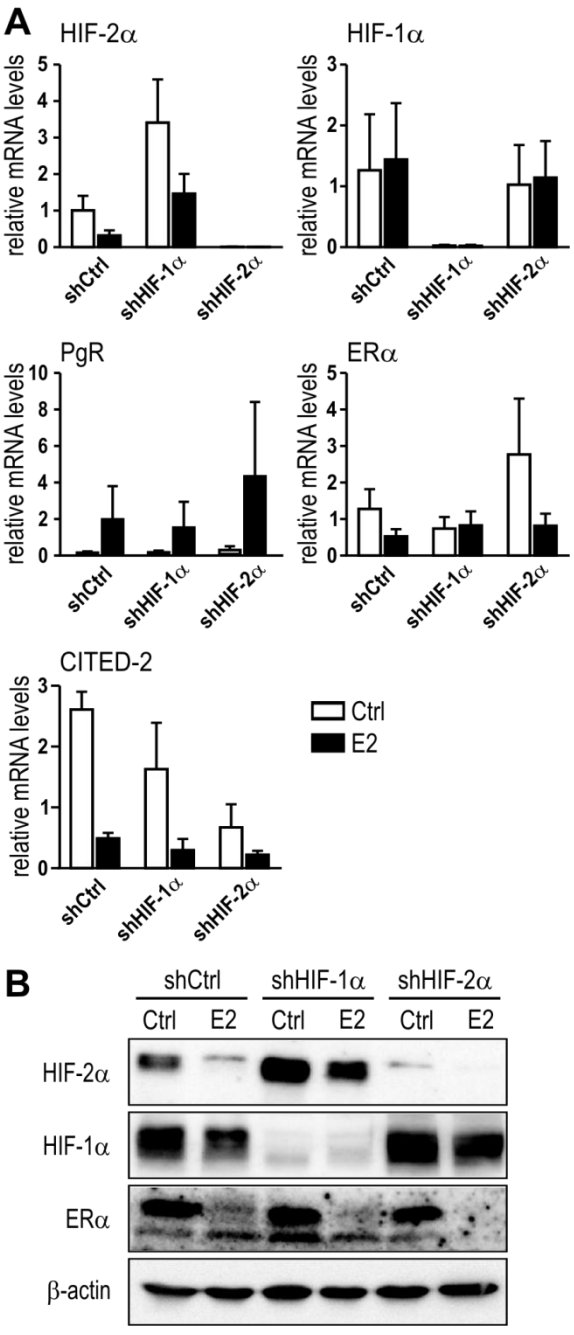

Figure 5: Mutual HIF-α inhibition is not involved in E2-dependent HIF-2α regulation. A. MCF-7 shCtrl, shHIF-1α and shHIF-2α cells were treated with 10 nM E2 for 24 hours under hypoxic conditions and mRNA levels determined by RT-qPCR. Shown are mean values ± SEM of three independent experiments. For statistical evaluation, the effects of E2 treatment were compared with control (Ctrl) treatment. **B.** Immunoblotting of MCF-7 cells treated with E2 as above.

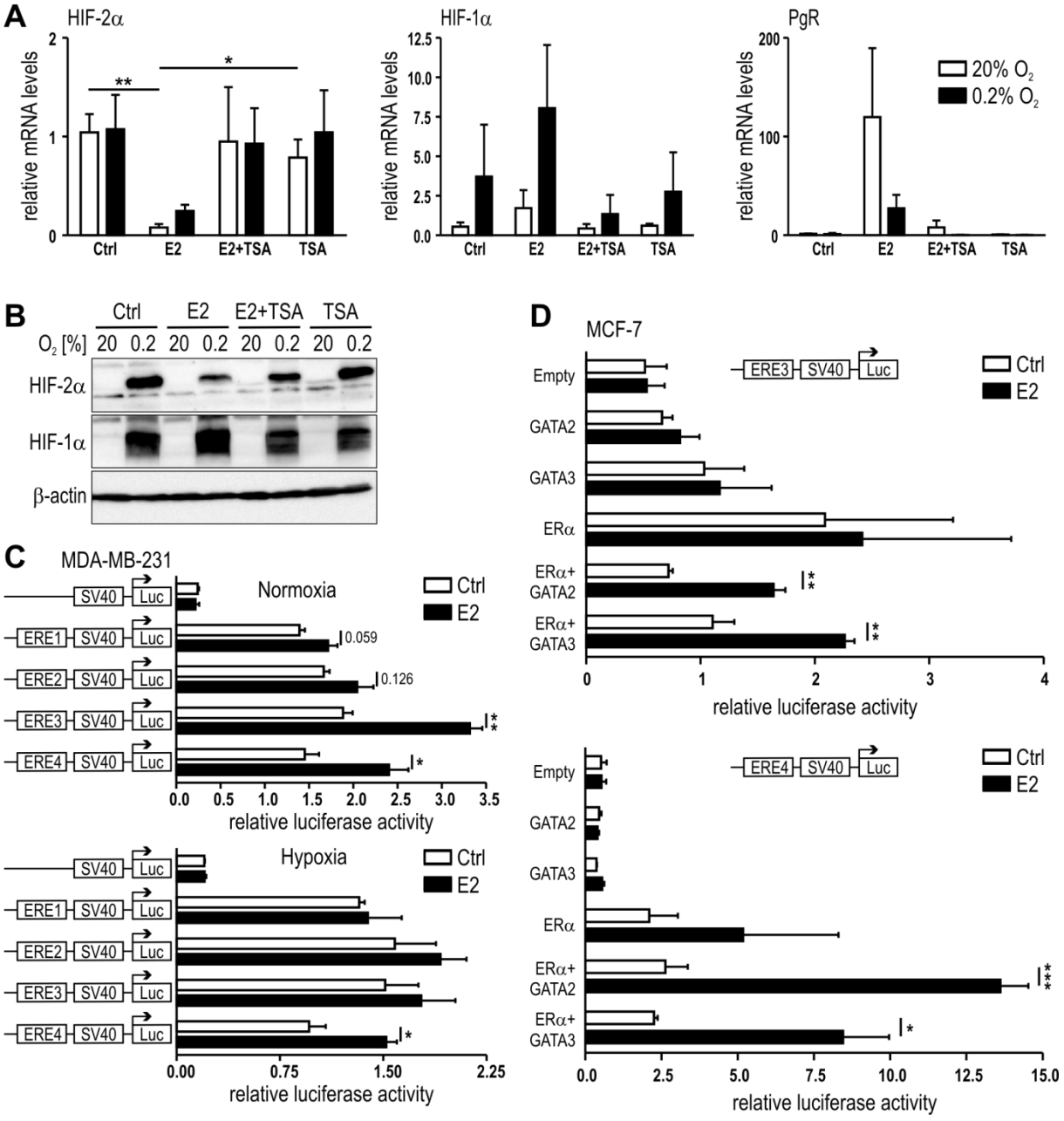

Figure 6: Role of histone deacetylation in E2-dependent HIF-2α regulation. MCF-7 cells were treated with 10 nM E2 with or without 250 nM trichostatin A (TSA), an inhibitor of HDAC. mRNA (**A**) and protein (**B**) levels were determined by RT-qPCR and immunoblotting, respectively. **C.** Dual luciferase reporter gene assays were performed with MDA-MB-231 cells transiently co-transfected with a constitutively expressed *Renilla* luciferase plasmid, an *EPAS1*-derived ERE1 to 4 driven firefly reporter gene plasmid, and an ERα overexpression vector. **D.** Luciferase reporter gene assays were performed with MCF-7 cells transiently co-transfected with a constitutively expressed *Renilla* luciferase plasmid, an *EPAS1*-derived ERE3 or ERE4 driven firefly luciferase reporter gene plasmids, and expression vectors for ERα, GATA-2 or GATA-3. Shown are mean values ± SEM of three independent experiments. For statistical evaluation, the effects of E2 without or with TSA treatment (**A**) were compared with control (Ctrl) treatment; the effects of E2 and TSA treatment were compared with E2

treatment. For dual-luciferase reporter gene assays (**C** and **D**), three independent experiments were performed in triplicates. The relative luciferase activity was obtained by dividing the firefly luciferase values by the corresponding *Renilla* luciferase values. **P*<0.05; ***P*<0.01; ****P*<0.001.

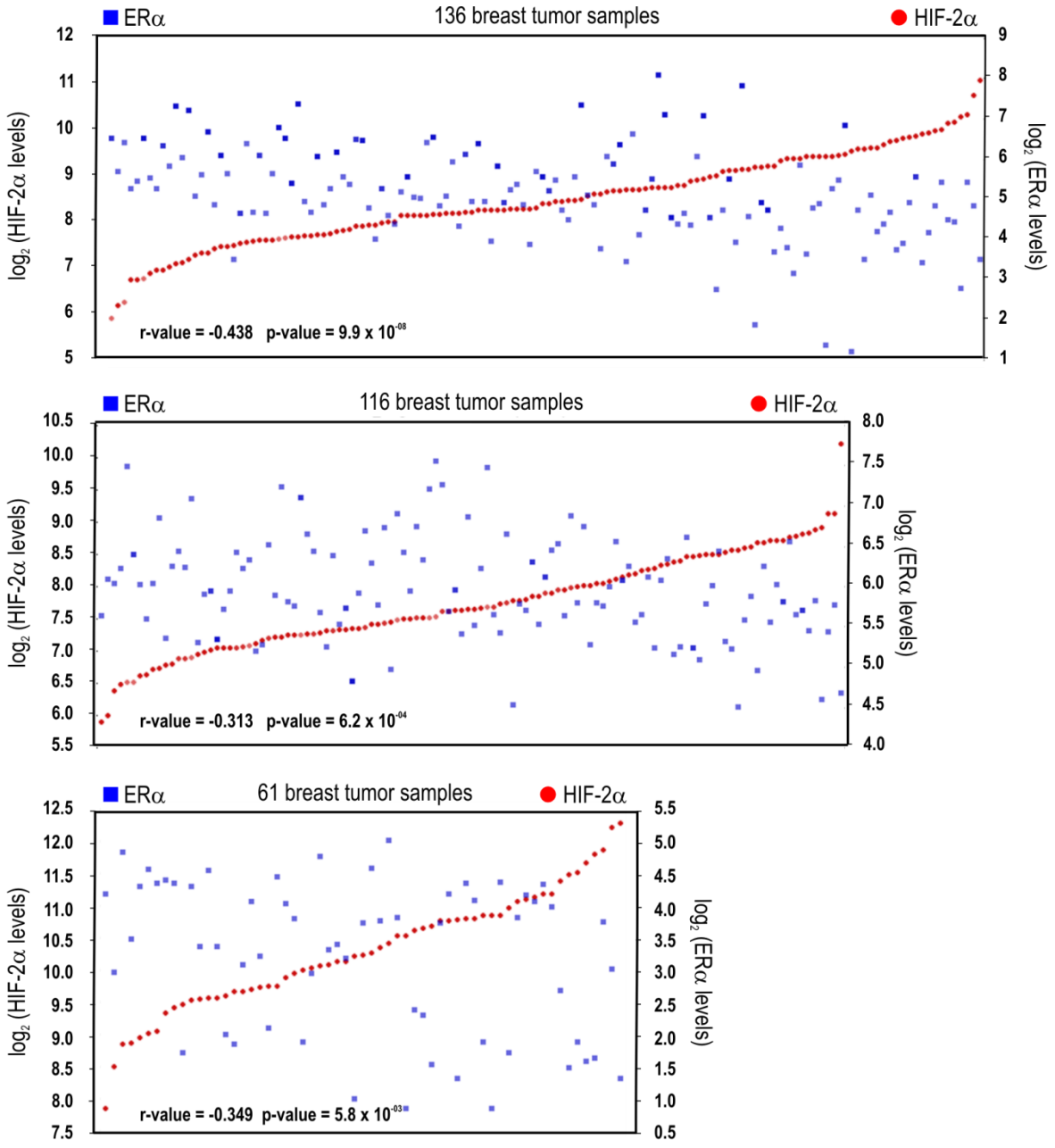

Figure S1: Negative association between ERa and HIF-2a in breast cancer. Microarray data from three independent studies were compiled using the R2 genomic analysis tool. HIF-2a mRNA levels negatively correlate with ERa levels in breast cancers in all studies analysed, as assessed by one-way ANOVA.

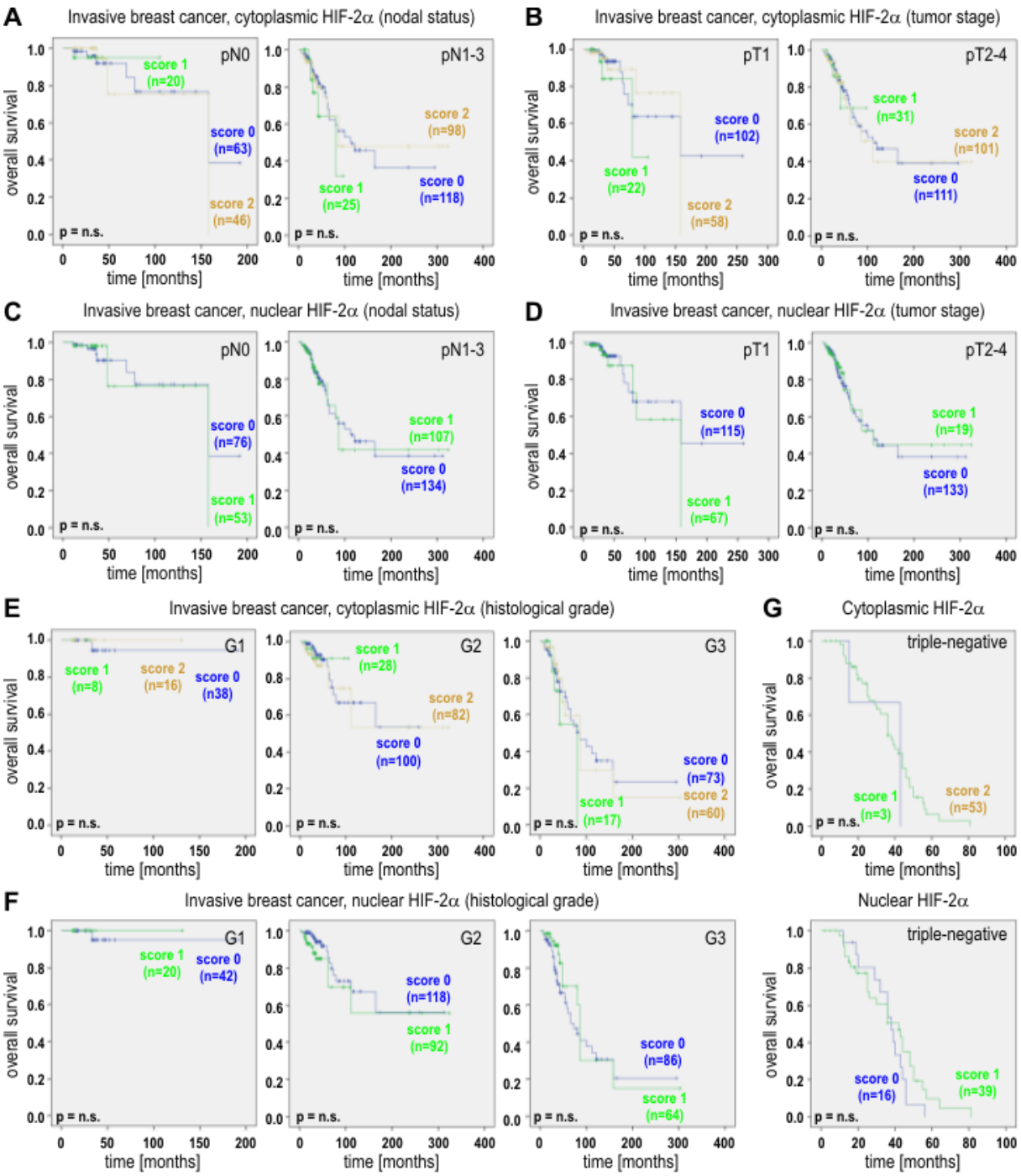

Figure S2: Kaplan-Meier survival curves related to HIF-2a scores. Kaplan-Meier survival curves of cytoplasmic (**A, B, E** and **G**) and nuclear (**C, D, F** and **G**) HIF-2a scores in breast cancer samples stratified according to nodal status (**A** and **C**), tumour stage (**B** and **D)**, histological grade (**E** and **F**), or in triple-negative breast cancer (**G**).

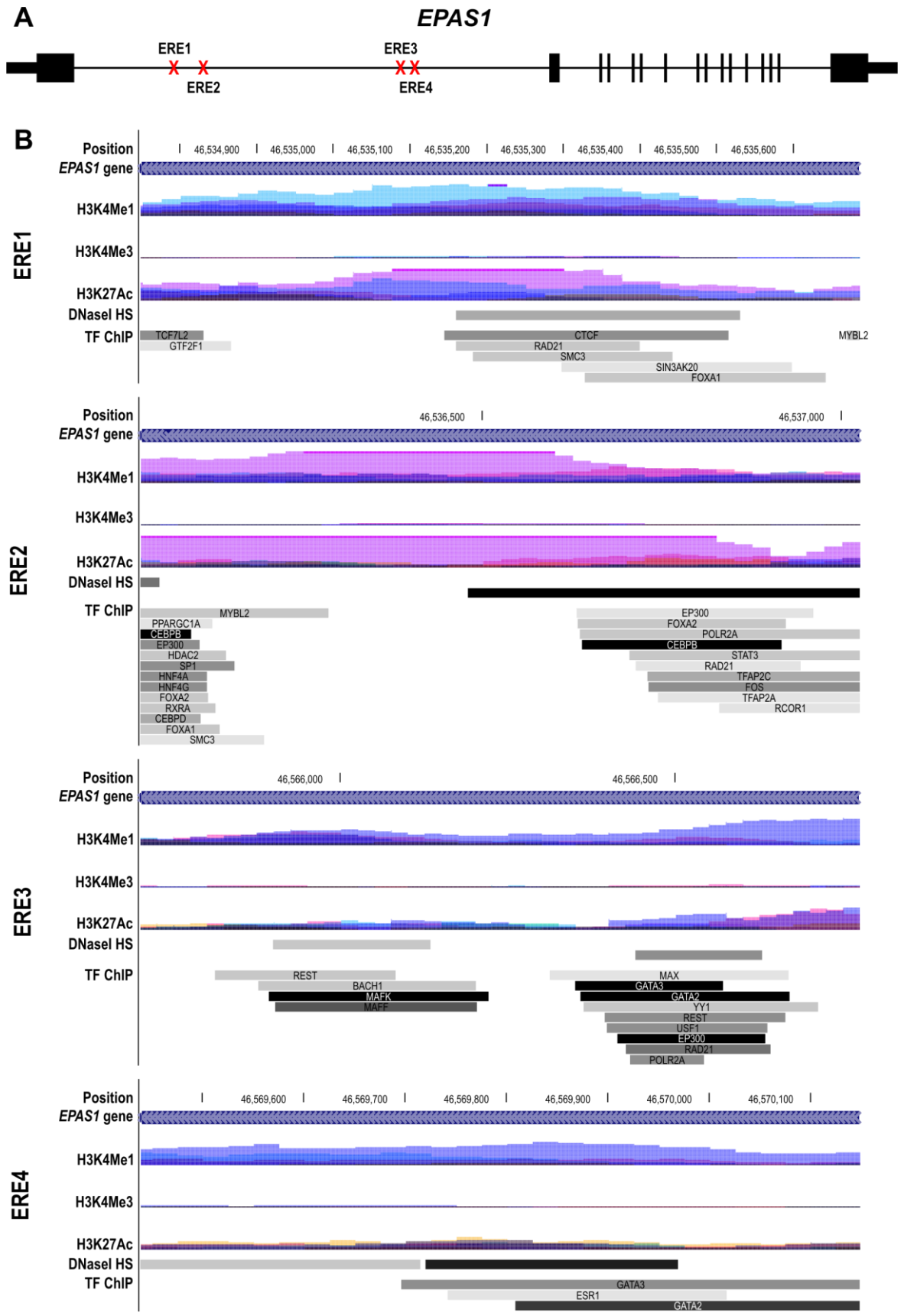

Figure S3: Potential ERE sites in *EPAS1* gene. A. Schematic representation of the *EPAS1* gene, indicating four estrogen response elements (EREs) located within the first intron. **B.** UCSC-integrated ENCODE data of the four ERE-containing loci (ERE1-4) of the *EPAS1* gene.

8. Discussion and final remarks

Breast cancer is one of the most common cancers and affects millions of women each year[1,2]. As a multifactorial disease, various factors influence the risk of breast cancer **(Table 8.1)**[9]. Molecularly, the involvement of both hypoxia and estrogen signalling pathways in the development and progression of breast cancer has been well established.

Table 8.1 Factors influencing breast cancer risk (adapted from [9]).

Factors that influence breast cancer risk
Genetic susceptibility and family history
Endogenous steroid hormone levels and exogenous hormone
Age at menarche and age and type of menopause
Parity and lactation
Life style, such as alcohol consumption and level of physical activity
Height, weight and body size

Among the HIF-α isoforms, HIF-1α is the more thoroughly investigated. Although HIF-2α has been long reported, there are still gaps regarding our understanding of the functions and regulations of HIF-2α in breast cancer. Therefore I aimed for the investigation of both the functions and modulations of HIF-2α by utilising breast cancer cell lines. Specifically, I aimed to:

1. Elucidate the regulation of WISP-2 by HIF-2α to reveal the role of HIF-2α in breast tumourigenesis. Furthermore, I aimed to explore the possible contributions of both HIF-2α and WISP-2 in the aggressiveness of breast cancer, and hence their contribution in overall survival in breast cancer patients.
2. Study the potential regulation of HIF-2α by estrogen signalling in ER-positive breast cancer cell lines.

8.1 HIF-2α-dependent regulation of WISP-2 and its effects on breast cancer progression

Immunohistochemical detection of HIF-2α, amphiregulin (AREG) and WISP-2 in tissue microarrays of 282 breast cancer patients with primary mammary carcinoma confirmed the higher abundance of AREG and WISP-2 in tumours associated with positive HIF-2α[161]. Furthermore, Kaplan-Meier survival curves revealed a significant survival benefit of patient subgroups expressing both AREG and WISP-2 at high levels[161]. Buoyed by the clinical evidence for a relation between HIF-2α and WISP-2, I decided to investigate further the molecular mechanism behind the regulation of WISP-2 by HIF-2α and the functional consequences of hypoxic WISP-2 regulation in breast cancer progression. Four hypoxia response elements (HREs) were discovered

within the *WISP-2* promoter region, in which two HREs were functional and were required for HIF-2α-dependent regulation of *WISP-2* promoter activity in MCF-7. Furthermore, the two active HREs are located within a microsatellite (MS) region which is highly polymorphic and has been established to play an essential role in tumour progression.

WISP-2 has been reported previously to correlate inversely with the aggressiveness of breast, pancreatic, colon and gastric cancer[171,173,293,294]. In breast cancer, WISP-2 shows transient overexpression during the progression of breast cancer; while it is not expressed in normal human mammary epithelial cells (HMECs), it is highly expressed in ER-positive non-invasive breast cancer cell lines, and is again undetected in triple-negative highly invasive breast cancer cell lines. The observations suggest the potential roles of WISP-2 as a tumour suppressor-like factor. Another prognostic marker for the progression of breast cancer is tumour macrophage infiltration. Immunohistochemical staining of 300 breast cancer samples with macrophage markers, CD68 and CD163, and pan-leukocyte marker CD45 was performed. Consistent with the higher survival benefit of WISP-2 expression in breast cancer patients, a negative correlation between WISP-2 and tumour macrophage numbers were observed, suggesting that HIF-2α-dependent expression of WISP-2 is a marker for better prognosis, due to the lower counts of macrophage infiltration and lower cancer aggressiveness.

Finally, in order to investigate the functional consequences of HIF-2α-dependent regulation of WISP-2, WISP-2 was knocked down (KD) in non-invasive MCF-7 cells and exposed to normoxia and hypoxia for different experimental settings. Proliferation of WISP-2-KD MCF-7 cells, but not HIF-2α-KD MCF-7 cells was significantly reduced under normoxia and hypoxia. Furthermore, the capability of HIF-2α-KD and WISP-2-KD MCF-7 cells to form colonies independently of anchorage was increased compared to control MCF-7 cells. Likewise, knockdown of both HIF-2α and WISP-2 resulted in a significant increase in the recovery of the scratch through a confluent cell layer. Interestingly both the anchorage-independent colony formation and scratch recovery of HIF-2α and WISP-2 knockdown were slightly reduced under hypoxia, which might be due to the residual WISP-2 induction under hypoxia. Together, these observations are consistent with a role of HIF-2α-dependent regulation of WISP-2 in cancer progression.

8.2 Estrogen-dependent regulation of HIF-2α in breast cancer

In breast cancer, cross-talk between hypoxia and estrogen signalling has been previously reported[232,233,237]. E2 treatment of ER-positive MCF-7 cells by our laboratory at different time-points resulted in the progressive down-regulation of HIF-2α mRNA levels under normoxia. To investigate the requirement for ER in the downregulation of HIF-2α, two luminal-like ER-positive (MCF-7, T-47D), one basal A-like triple-negative (MDA-MB-468), and one basal B-like triple-negative (MDA-MB-

231) breast cancer cell line were treated with E2, and HIF-2α mRNA and protein levels were investigated. Consistent with our hypothesis that the downregulation of HIF-2α by E2 is ER-dependent, HIF-2α mRNA and protein levels were downregulated upon treatment with E2 in ER-positive cell lines and the downregulation is completely absent in triple negative cell lines. Subsequent treatments of MCF-7 cells with ERα-specific (PPT) and GPR30-specific (G1) agonists further confirmed the role of ERα in HIF-2α downregulation.

To examine the clinical relevance of ERα-dependent HIF-2α regulation, tamoxifen is used to antagonise the effect of E2 on MCF-7 cells. Tamoxifen, a selective estrogen receptor modulator (SERM) is currently used as an adjuvant to chemotherapy on ER-positive breast cancer patients. Due to its extraordinary efficacy against breast cancer mortality, it is currently recommended by the World Health Organisation (WHO) as the treatment of choice for estrogen-sensitive breast cancer. In our laboratory, treatment of E2 in the presence of tamoxifen results in diminished HIF-2α downregulation, giving a hint about the clinical importance of ERα-dependent HIF-2α regulation in breast cancer progression. It would be fascinating to look at the changes in the level of HIF-2α in samples from breast cancer patients. Unfortunately, we cannot compare between the experimental protocol we used for our treatment of breast cancer cell lines and the current treatment protocol on breast cancer patients. In our treatment protocol, we treated MCF-7 cells with E2 with or without tamoxifen before quantifying the changes in mRNA and protein levels. However, in a clinical setting, once a patient is diagnosed with breast cancer, a surgery was performed to remove the tumour. After the ER-status of the breast cancer has been established, then treatment either with or without tamoxifen can proceed. As such, it is not possible for us to compare the two different experimental settings and hence our lack of data in confirming our *in vitro* observations.

Interestingly, when ERα was knocked-down in MCF-7, HIF-2α mRNA levels increased significantly, further indicating the modulation of HIF-2α transcription by ERα even in the absence of E2. Similarly, an increase in HIF-2α mRNA and protein levels can be observed even though this was not statistically significant. Analysis of gene microarray data from various breast cancer studies showed similar inverse correlation between the mRNA levels of HIF-2α and ERα in breast cancer patients as we found in our cell line study. To elaborate the clinical relevance of this modulation, samples from breast cancer patients were investigated based on the ERα and HIF-2α protein levels against the survival of the patients. Additionally, samples were stratified based on ER and HER2 expression, nodal status, tumour stage, and histological grade. Differing from our previous report, no significant survival benefit was observed in breast cancer patients expressing higher levels of HIF-2α[161]. The previous study used 282 samples from patients with primary mammary carcinoma without further stratification of the samples into different categories of breast cancer. In the current study, 690 breast cancer patients with primary mammary carcinoma were used. In addition, the samples were mixed population and were classified into

ER-positive, ER-positive HER-2-positive, and triple-negative breast cancers. As such, the latter study included more information and provided better statistical power for our calculation at the survival benefit. In fact, stratification of HIF-2α nuclear expression according to HER2 status of patients displayed a significant survival benefit for ERα and HER-2-positive patients. Intriguingly, treatment of E2 on BT474, a triple-positive breast cancer cell line, showed less downregulation of HIF-2α compared to treatment of ER-positive breast cancer cells, in line with a role of HIF-2α as gene associated with favourable prognosis.

Mechanistically, mutual HIFα inhibition is not responsible for the regulation of HIF-2α by ERα. Results from this study suggest a transcriptional modulation of HIF-2α by ERα in the presence and absence of E2. One of the means of gene regulation is by chromatin remodelling, in which histone deacetylation is one potential mechanism. Inhibition of HDACs prevented the downregulation of HIF-2α by E2, indicating the role of histone deacetylation in the observed modulation of HIF-2α. Moreover, dysregulation of HDACs had been reported in numerous cancer cases, including in breast cancer. To further explore the potential mechanism, *EPAS1* was screened for the presence of EREs. Four EREs were discovered within the first introns of *EPAS1*, in which two EREs; ERE3 and ERE4 were found to be active, particularly in the presence of GATA-2 and GATA-3. Activation of ERα by E2 results in the translocation of the receptors into the nucleus, where the E2-ERα dimer complex recruits co-regulators, which include possibly GATA-2, GATA-3 and HDACs. Histone deacetylation due to the recruited HDACs causes the remodelling of chromatin and subsequently repression of HIF-2α transcription. Further studies are warranted for the elaboration of the mechanism behind HIF-2α regulation.

One question that arose from this study is which type of HDAC is involved in the deacetylation of *EPAS1*. HDACs can be classified into three classical classes (I, II, and IV) and consist of total 11 members. Another class of HDACs, class III or sirtuins are unique due the possession of NAD-dependent catalytic sites and resistant to HDAC inhibitors. HDACs have been reported to be involved in tumourigenesis[284,285,295]. Class I HDACs: HDAC-1, -2, and -3 are especially often overexpressed in human tumours, including breast tumours, and knockdown of HDAC-1 and -2 has been shown to be sufficient to reduce tumour growth *in vivo*[285,296,297]. It will be interesting to distinguish which HDAC is involved in HIF-2α downregulation and if the implicated HDAC is also linked to breast cancer progression. One prime candidate HDAC is HDAC7. Previously, it has been reported that estrogen repression of RPRM (repression of reprimo), a cell cycle inhibitor, requires interaction between ERα, HDAC7 and FoxA1[286]. HDAC7, a class II HDAC, binds to ERα and represses ERα's transcriptional activity without deacetylation[286]. Additionally, class II HDACs have been shown to regulate transcription by interacting with a complex containing HDAC3 and the transcriptional corepressor NCoR/SMRT, providing another possible way in which ERα activation represses transcription of HIF-2α[295]. Pharmacological approaches to inhibit class II HDAC are limited due to

the fact that currently no class II-specific HDAC inhibitor exists. One possible approach is to specifically knockdown members of class II HDAC to identify the HDACs involved in HIF-2α regulation.

Another question is the role of GATA-2 and GATA-3 in the regulation of HIF-2α. ERE4 has been identified using UCSC genome browser to be bound by ERα in both MCF-7 and T-47D[195]. Furthermore, from the same genome browser, ERE4 is also the site of binding for GATA-2 and GATA-3. As such, ERE4 is assumed to be the primary target of ERα binding upon treatment with E2. Indeed, luciferase reporter assays showed induction of activity upon treatment with E2 in the presence of both ERα and GATA-2 or GATA-3. However, this did not explain the downregulation of HIF-2α mRNA levels and protein levels. One explanation is the artificial setting of the assay, in which a short string of DNA containing ERE4 was expressed together with ERα and GATA-2/3. It is now well established that gene regulation is a dynamic event, involving multiple factors and looping of downstream or upstream DNA. Whereas luciferase reporter assays represent a powerful tool to study gene regulation, expression of a non-chromatinised DNA region only containing ERE4 does not fully reflect *in vivo* regulation of *EPAS1*. Our results however prove the functionality of ERE4 in regards to ERα, GATA-2 and GATA-3 binding. To circumvent the limitation of luciferase reporter assays, GATA-2 or GATA-3 could be knocked down in ERα-deficient MCF-7, followed by treatment with E2. In the absence of ERα and GATA-2/3, it is expected that HIF-2α levels will increase and the downregulation in the presence of E2 will be blunted. Moreover, the CRISPR-Cas method can be used to mutate the ERE4 within *EPAS1* followed by treatment with E2. Destruction of ERE4 within *EPAS1* will result in complete exclusion of ERα binding upon E2 activation, and hence elimination of HIF-2α regulation.

8.3 Final remarks

Separately, hypoxia and estrogen play important role in breast cancer development. Unsurprisingly, the two distinct signalling pathways might cross-path and affect each other. In this thesis, I have studied both the functional consequences and regulation of HIF-2α in breast cancer. The results from the study of the effects of estrogen-dependent regulation of HIF-2α prompted me to propose a potential mechanism behind the observed modulation **(Figure 8.1)**.

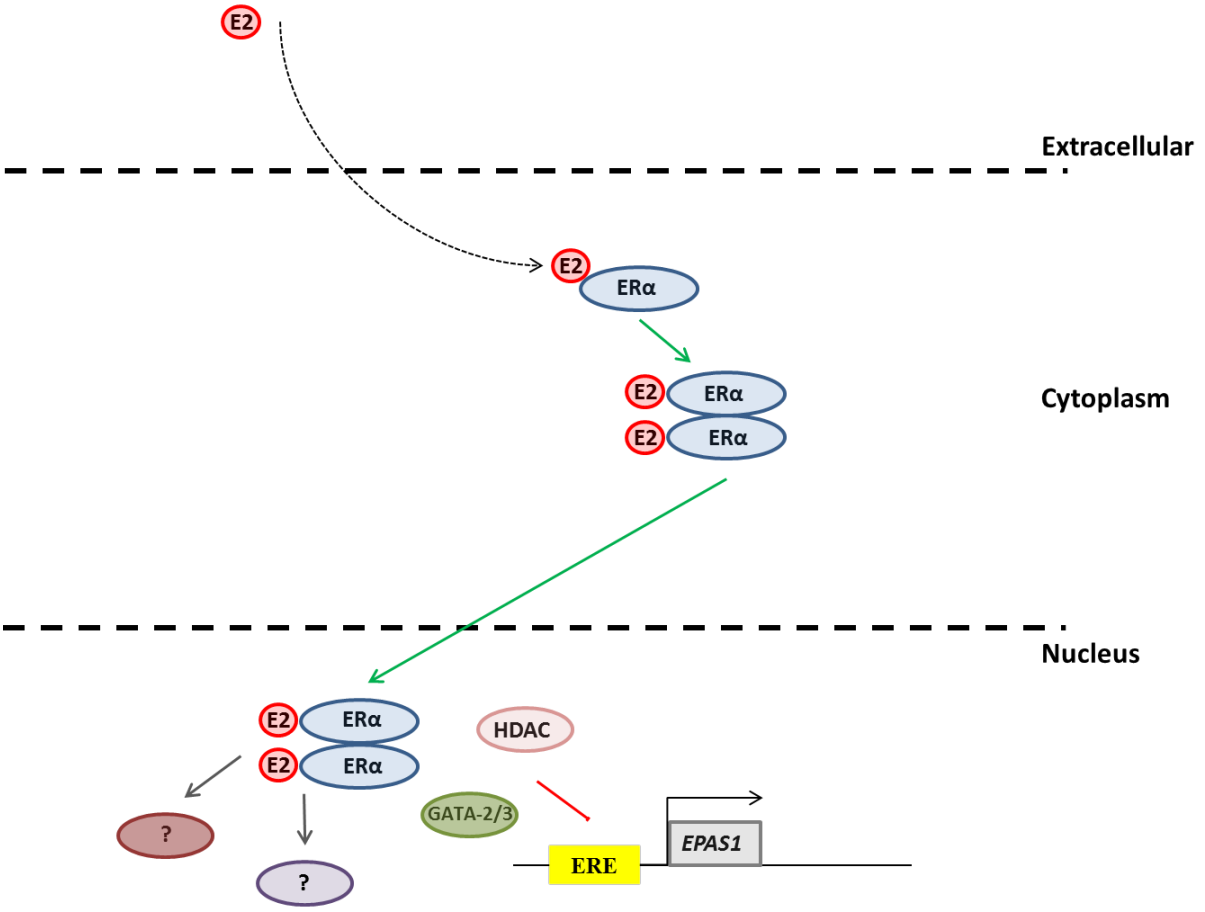

Figure 8.1 The proposed model for the E2-dependent HIF-2a regulation. Activation of ERa by E2 results in translocation of the activated ERa homodimer to the nucleus. Once in the nucleus, the activated receptors recruit co-regulators, including GATA-2/3 and HDAC, which subsequently deacetylate the down-stream target gene (*EPAS1*) and repress the transcription of the target gene.

In summary, my thesis reports a novel regulation of HIF-2α by estrogen signalling, which might partially explain the association of high HIF-2α in HER2-breast cancer patients with better prognosis. The importance of both estrogen signalling and HIF-2α in breast cancer development suggests the possibility of HIF-2α levels and ERα status as prognostic markers in breast cancer. Furthermore stabilisation of HIF-2α or maintenance of HIF-2α levels might be a treatment option for ER-positive breast cancer patients.

8.4 References

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Contributions to publications and manuscripts

1. **Fuady, J. H.**, Gutsche, K., Santambrogio, S., Varga, Z., Hoogewijs, D., and Wenger, R. H. (2015) Estrogen-dependent downregulation of HIF-2a in invasive breast cancer cells. ***Submitted to Oncotarget****.*
   All figures

2. **Fuady, J. H**., Bordoli, M. R., Abreu-Rodriguez, I., Kristiansen, G., Hoogewijs, D., Stiehl, D. P., and Wenger, R. H. (2014) HIF mediated induction of WISP-2 contributes to attenuated breast cancer progression. ***Hypoxia,*** 2:23-33.
   Figure 1B and subfigure 1D
   Figure 5A-E

3. Vinogradov, S. N., **Fuady, J. H.,** Roy, S. W., Hankeln, T., and Hoogewijs, D. Diversity of nematode globins, a major update. ***In preparation****.*
   Figure 3
   Table 1-3

4. Muller, C., **Fuady, J. H.,** Hoogewijs, D., Wenger, R. H., Ruegg, C., and Zweifel, M. Expression of soluble and transmembrane VEGF receptor 1 in breast cancer and endothelial cells under normoxia and hypoxia. ***In preparation.***
   Subfigure 2

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University of Zurich, Institute of Physiology
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Research internship
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Bachelor of Science in Biotechnology
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Research internship
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Teaching

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- Assistant in BIO401 courses for biology master students (2012-2015)

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  Zürich, Switzerland, August 2015
- Joint cancer meeting – **oral presentation**
  Zürich, Switzerland, June 2015
- Keystone symposia – **poster presentation**
  Hypoxia: from basic mechanisms to therapeutics
  Dublin, Ireland, May 2015
- 93rd annual meeting of the German Physiological Society – **poster presentation**
  Mainz, Germany, March 2014
- 8th retreat of the PhD prorgramme in Integrative Molecular Medicine (imMed) – **oral presentation**
  Mariastein, Switzerland, May 2013
- 8th ZIHP symposium – **poster presentation**
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- 7th retreat of the PhD programme in Integrative Molecular Medicine (imMed) – **poster presentation**
  Stein am Rhein, Switzerland, June 2012

Publications

1. **Fuady, J. H.,** Gutsche, K., Santambrogio, S., Varga, Z., Hoogewijs, D., and Wenger, R. H. (2015) Estrogen-dependent downregulation of HIF-2α in invasive breast cancer cells. ***Submitted to Oncotarget.***
2. Fuady, J. H., Bordoli, M. R., Abreu-Rodriguez, I., Kristiansen, G., Hoogewijs, D., Stiehl, D. P., and Wenger, R. H. (2014) HIF mediated induction of WISP-2 contributes to attenuated breast cancer progression. ***Hypoxia,*** 2:23-33.
3. Vinogradov, S. N., **Fuady, J. H.,** Roy, S. W., Hankeln, T., and Hoogewijs, D. Diversity of nematode globins, a major update. *In preparation.*
4. Muller, C., **Fuady, J. H.,** Hoogewijs, D., Wenger, R. H., Ruegg, C., and Zweifel, M. Expression of soluble and transmembrane VEGF receptor 1 in breast cancer and endothelial cells under normoxia and hypoxia. *In preparation.*

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